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Real-time activation profiles of single T cell arrays following controlled interaction with antigen-presenting cells

by

Anna Desalvo

Thesis for the degree of Doctor of Philosophy

May 2019

Supervisors: Prof Tim Elliott

Prof Hywel Morgan

Dr Edd James
ABSTRACT

The aim of this project was to develop a new microdevice to allow precise control of cell-cell interactions, and to apply it to measure T cell signalling in arrays of single cells in real-time after stimulation by live antigen presenting cells.

The specific T cell responses which underlie effective immunotherapy of cancer are mediated via cell-cell interactions and the formation of immune synapses. However, methods to study these interactions are limited by lack of control over single cell pairing and analysis methods. This project describes a new approach to track the activation profile of cytotoxic T cells against tumour cells in a high-throughput manner.

A simple yet effective mechanism to pair the single T cells with antigen presenting cells was optimized to study cell-cell contact in a time-controlled manner. The cell-trap array is an open system consisting of thousands of microwells cast in an agarose block, which is biocompatible and permeable to nutrients allowing functional observations on viable cells to be carried out over a number of hours. T cells can be singularly isolated in the wells via passive sedimentation and size exclusion, achieving up to 90% occupancy.

Activation profiles of thousands of single CD8+ cells could be screened in the same field of view using calcium-chelating fluorophore dyes to measure the early stages of T cell signalling. Custom software was developed to process experimental data and allow rapid detection of individual cells with the strongest activation signals defined by several different criteria. This capability could be used in future to identify and characterise single T-cells that have especially high propensity to be activated by immunotherapy such as vaccination or checkpoint blockade, in preclinical animal models and biopsy samples from human cancer patients.
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Data for "Real time activation profiles of single T cell arrays following controlled interaction with antigen-presenting cells"

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Research Thesis: Declaration of Authorship

Print name: ANNA DESALVO

Title of thesis: “Real-time activation profiles of single T cell arrays following controlled interaction with antigen-presenting cells”

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:


   - Desalvo A., Bateman F., James E., Morgan H., Elliott T., “Stimulation of Single T Lymphocytes Isolated in Microwell Arrays Using a Pressurized Membrane”, Microfluidics Analytical Chemistry Conference 1st February 2018, National Oceanography Centre Southampton, United Kingdom

Signature: ................................................................. Date: 26/10/2019
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## Definitions and Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ACT</td>
<td>Adoptive cell transfer.</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy.</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B (PKB).</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1.</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell.</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate.</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve.</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin.</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium.</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor.</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation.</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DeoxyriboNucleic Acid.</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide.</td>
</tr>
<tr>
<td>C-PC</td>
<td>C-Phycocyanin.</td>
</tr>
<tr>
<td>CPRG</td>
<td>ChloroPhenylRed-D-Galactopyranoside.</td>
</tr>
<tr>
<td>CRACC</td>
<td>Calcium Release Activated Calcium Channel.</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes.</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte Antigen-4.</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell.</td>
</tr>
<tr>
<td>DEP</td>
<td>Dielectrophoresis.</td>
</tr>
<tr>
<td>DI</td>
<td>De-ionised.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DMF</td>
<td>N,N-DiMethylFormamide.</td>
</tr>
<tr>
<td>DMSO</td>
<td>DiMethyl SulfOxide.</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid.</td>
</tr>
<tr>
<td>DOP-PCR</td>
<td>Degenerate Oligonucleotide Primed – Polymerase Chain Reaction.</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phospate-Buffered Saline.</td>
</tr>
<tr>
<td>EDTA</td>
<td>EthyleneDiamineTetraacetic Acid.</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein.</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum.</td>
</tr>
<tr>
<td>EWOD</td>
<td>Electrowetting on dielectric.</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting.</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum.</td>
</tr>
<tr>
<td>FC</td>
<td>Flow Cytometry.</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum.</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorescein di(β-D-galactopyranoside).</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence In Situ Hybridization.</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorescence (channel).</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter.</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein IsoThioCyanate.</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.</td>
</tr>
<tr>
<td>ICI</td>
<td>Immune Checkpoints Inhibitor.</td>
</tr>
<tr>
<td>IL</td>
<td>Inter Leukin.</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate.</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol triPhosphate Receptor.</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol.</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motif.</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for Activation of T cells.</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte Function-Associated antigen 1.</td>
</tr>
<tr>
<td>LCK</td>
<td>Lymphocyte-specific protein tyrosine Kinase.</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser Capture Microdissection.</td>
</tr>
<tr>
<td>MALBAC</td>
<td>Multiple Annealing and Looping–Based Amplification Cycles.</td>
</tr>
<tr>
<td>MDA</td>
<td>Multiple Displacement Amplification.</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity.</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Hystocompatibility Complex.</td>
</tr>
<tr>
<td>MNP</td>
<td>Magnetic Nanoparticle.</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian Target Of Rapamycin Complex 1.</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight.</td>
</tr>
<tr>
<td>nDEP</td>
<td>Negative DiElectroPhoresis.</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T-cells.</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor k-light-chain-enhancer of activated B cells.</td>
</tr>
<tr>
<td>NGS</td>
<td>Next Generation Sequencing.</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin.</td>
</tr>
<tr>
<td>PA</td>
<td>Polyacrylamide.</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrilamide Gel Electrophoresis.</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline.</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction.</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell Death protein 1.</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene Oxide.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate.</td>
</tr>
<tr>
<td>PPO</td>
<td>Polypropylene Oxide.</td>
</tr>
<tr>
<td>pDEP</td>
<td>Positive DiElectroPhoresis.</td>
</tr>
<tr>
<td>PDMS</td>
<td>PolyDiMethylSiloxane.</td>
</tr>
<tr>
<td>PEB</td>
<td>Post Exposure Bake.</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol.</td>
</tr>
<tr>
<td>PI-3</td>
<td>PhosphoInositide-3.</td>
</tr>
<tr>
<td>PIP2</td>
<td>PhosphoInositol BioPhosphate.</td>
</tr>
<tr>
<td>PLC</td>
<td>PhosphoLipase C.</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate).</td>
</tr>
<tr>
<td>PKB (Akt)</td>
<td>Protein Kinase B.</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C.</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative (real-time) Polymerase Chain Reaction.</td>
</tr>
<tr>
<td>pMHC</td>
<td>Peptide-Major Hystocompatibility Complex.</td>
</tr>
<tr>
<td>RER</td>
<td>Rough Endoplasmic Reticulum.</td>
</tr>
<tr>
<td>RIPA</td>
<td>RadioImmunoPrecipitation Assay.</td>
</tr>
<tr>
<td>RNA</td>
<td>RiboNucleic Acid.</td>
</tr>
<tr>
<td>ROI</td>
<td>Region Of Interest.</td>
</tr>
<tr>
<td>SC</td>
<td>Single Cell.</td>
</tr>
<tr>
<td>scWB</td>
<td>Single Cell Western Blot.</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation.</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate.</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis.</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/Endoplasmic Reticulum Calcium transport ATPase.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SHL8</td>
<td>SIINFEHL (peptide).</td>
</tr>
<tr>
<td>SL8</td>
<td>SIINFEKL (peptide).</td>
</tr>
<tr>
<td>SOC</td>
<td>Store Operated Calcium channel.</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter.</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA.</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with Antigen Processing.</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor.</td>
</tr>
<tr>
<td>TCR:pMHC</td>
<td>T Cell Receptor:Peptide-Major Hystocompatibility Complex.</td>
</tr>
<tr>
<td>TEA</td>
<td>TriEthylAmine.</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine.</td>
</tr>
<tr>
<td>TG</td>
<td>ThapsiGargin.</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate-conjugated.</td>
</tr>
<tr>
<td>UV</td>
<td>UltraViolet.</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot.</td>
</tr>
<tr>
<td>WGA</td>
<td>Whole Genome Amplification.</td>
</tr>
<tr>
<td>WTA</td>
<td>Whole Transcriptome Amplification.</td>
</tr>
<tr>
<td>ZAP70</td>
<td>Zeta-chain-Associated Protein kinase 70.</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

This Chapter will introduce the context, aims and scope of the present project. It will list the goals and achievements obtained throughout the work. The thesis structure will be detailed, to guide the reader in the understanding of the thesis.

1.1 Context

The project development was driven by the need of biologists for a platform that would allow screening of large numbers of single lymphocytes to gain insights into their activation mechanisms.

When a pathogen invades the human body, Antigen Presenting Cells (APCs) degrade its component proteins and load peptide fragments, called epitopes, onto newly synthesised Major Histocompatibility Complex (MHC) molecules. The resulting peptide:MHC (pMHC) complexes are then transported to the cell surface, where they can be recognized by specific CD8+ cytotoxic T lymphocytes (CTL). Upon contact with APCs loaded with pathogenic antigens, these lymphocytes become activated, and can release granules containing cytotoxins into the infected cells, killing them thereby preventing the spread of the infection to neighbouring cells. Cytotoxic T cells have very specific receptors, that can recognize precise epitopes only in combination with MHC. Immunodominance is the hierarchical response of the lymphocytes when different antigenic epitopes are displayed, and limits the breadth of multiple immune responses. In fact, some epitopes that are known to activate specific T cells, don’t work when combined with other peptides.

The mechanisms that define this hierarchy are not yet understood, also due to the lack of technologies that could enable the study of T lymphocytes activations at a single cell level. Cell diversity is logically expected amongst T cells, that need to be individually specific to designated peptides among a wider range of epitopes, and downscaling the data analysis to a single-cell resolution becomes essential with the scope of finding the underlying mechanisms of immunodominance.

In our perspective, in order to study why certain cells are more efficient in eradicating diseases, it is necessary to analyse multiple single cell-cell interactions and follow single T lymphocyte early activations over time, since our belief is that the hierarchy might be dictated by the first T Cell Receptor : peptide-loaded MHC (TCR:pMHC) ligation (first minutes following the contact).
1.2 Aims and scope

The end goal of this project is to build a platform that could be used by biologists to study in detail lymphocyte activation mechanisms at the single cell level, and gain insight into the immunodominant subpopulation of T cells, yielding information that could be used to modify and target the patient’s immune response.

The project objective is to design a microfluidic device able to trap single T cells and pair them with APCs, measure CTL activation over time and enable downstream (single-cell) biochemical analysis such as RNAseq, western blot and cytokine profiling. The analysis should be high-throughput, and allow monitoring of thousands of T cells in one go. Lymphocytes and APCs should be paired simultaneously, hence the device should allow to control of the time and duration of the cell-cell contact.

The project started and developed in an interdisciplinary way, having the first goal of defining and building the platform for single cell handling, and the parallel requirement to validate a biological model to be used to test the platform. For this reason, the present work is the result of a collaboration between the Faculties of Medicine (Cancer Research, Immunology) and Engineering (Electronic and Computer Sciences, Hybrid Biodevices).

1.3 Summary of main achievements

This thesis describes a new and straightforward method to pair single lymphocytes to APCs.

After successfully validating a biological cell model to adopt for the testing of the platform, the device that allows isolation of thousands of single T cells in an open system is presented. Being transparent, the plate can be used both with upright and inverted microscopes; it is fabricated with a hydrogel, making it biocompatible and permeable to cells nutrients and stimulants. High occupancies guarantee the screening of thousands of single CD8\(^+\) cells within one field of view, and stimulation of the lymphocytes with soluble reagents proved that activation could be recorded and trends were consistent with the time responses gathered using bulk population studies via flow cytometry.

More importantly, a simple yet effective mechanism to pair the single T cells with APCs has been optimized, and biological activation was recorded. This is the first open platform where thousands of cell-cell contacts can be induced simultaneously and monitored over time to our knowledge, making it appealing for a variety of biological applications, that go beyond the specific goal.
A customized code that relies on ImageJ and Matlab softwares was developed to rapidly post-process single cell activations, define the top responders and trace them back to the trapping plate.

1.4 Thesis structure

The dissertation is divided in seven Chapters. Following this Introduction, the subsequent topics will be developed.

Chapter 2: is a literature review that covers the two main aspects underlying the present work: the biology of T cell activation and devices that allow single cell studies.

Chapter 3: summarises the materials and standard protocols used throughout the study.

3.5 covers the model optimization. Starting from the cell line selection and maintenance of a sensitive population of lymphocytes, it treats their chemical activation with ionomycin and biological activation using flow cytometry with antibodies to the T cell receptor complex (antiCD3) or via APCs loaded with immunogenic peptides. Aspects such as the relevance of the buffer solution and the force in the cell-cell interaction are considered.

Chapter 5: introduces the trapping device that allows isolation of thousands of single cells. An overview on the microwell platform design and fabrication, material selection, trapping efficiencies and cell viability follows. Cell stimulation protocols using soluble stimulants (ionomycin, antiCD3) is detailed and supported by experimental data, and the code to analyse the single cell responses is explained.

Chapter 6: details the development of the cell-cell interaction setup, starting from an existing platform and arriving at the final device. An initial approach using nanoparticle-loaded APCs to bring the cell pairs into contact magnetically, are reported, and a new version of pairing platform is introduced, where APCs are grown confluent onto flat surfaces that are brought into contact with the traps array of lymphocytes. Methods to pattern the APCs are discussed, with potential of implementation into the pairing system, and a microfluidic device to create cell-cell contact is introduced, as an alternative to the current setup.

Chapter 7: summarises the conclusions from this work, and suggests possible future directions. Specifically, on-chip analysis using single cell western blot technique and cell retrieval using a micromanipulator are suggested for further developments of the platform.
Chapter 2: Literature review

This chapter has the aim of giving the reader all the background information required to have a complete understanding of the project goals, and to familiarise with the literature underlying this topic, both under the immunological and engineering points of view.

At first, the T lymphocyte activation process will be treated more in detail. This should highlight the biological targets that will be addressed in the development of the device. The final part of this section will remark the contribution that this study could give to the current cancer immunology research.

Secondly, a literature overview of the existing technologies to study single cells will be presented. The intended purpose is to show the rationale behind the choice of using microchambers to capture individual cells.

2.1 T cell activation and immunodominance

When our body is invaded by pathogens, these are processed by Antigen Presenting Cells (APCs), that degrade their proteins into sequences of 8-11 aminoacids called epitopes. Once the peptides enter the Rough Endoplasmic Reticulum (RER), they are associated with the Major Histocompatibility Complex Class I (MHC1). This combination (pMHC) is subsequently exposed to the APCs cell surface (schematic in Figure 2.1, a). An APC displays 1-100,000 of different antigens on its cell surface1–6.

Our immune system poses two barriers against invading pathogens. At first, the innate immune system acts as a non-specific barrier; once the pathogen is recognized, a more specific yet slower response to the infection is given by the adaptive immune system. This second response is carried out by T lymphocytes, white blood cells that can be divided in two classes: CD4+ and CD8+. The former is a population of helper cells secreting cytokines that activate B cells, while CD8+ (Cytotoxic T Lymphocytes, CTL) can eradicate the infection releasing cytotoxins into the pathogenic cells after ligation. CTLs recognize the pMHC complexes on the APCs via their T Cell Receptor (TCR). When a cytotoxic T cell contacts an APC, an immunological synapse between the cells is created, and a cascade of phosphorylation reactions ensue in the CD8+, initiating activation7,8.

While the cascade will be detailed in Paragraph 2.1.1, it is important to mention at this point that an early response to T cell activation is associated with an increase in the intracellular levels of Ca2+, due both to the release from internal stores and to the opening of calcium
channels (Calcium Release Activated Calcium Channels, CRACC) subsequent to the stores depletion. Mitochondria sequester the excess of Ca\textsuperscript{2+}, sustaining the activation response for longer times\textsuperscript{9–12}. The increase in cytosolic calcium can thus be used to assess T lymphocyte activation after TCR:pMHC engagement, by staining the T cells with calcium-dependent fluorophores (schematic in Figure 2.1, b).

Figure 2.1 Schematics of the CTL-APC interaction. Fig a. shows the mechanism leading to antigen presentation in the APC; the main steps can be summarized with the proteasomal degradation of the pathogen, the binding of its epitopes to MHC1 complexes and the exposure of the pMHC1 molecules on the cell surface. Fig b. represents the early stages of T cell activation, involving an increase of intracellular calcium (represented by the red circles), both released from the internal stores and uptaken via CRACC channels. Loading the lymphocytes with a fluorescent probe for intracellular calcium (pictured with the grey triangles), it is possible to assess their activation state using microscopy or flow cytometry, and to monitor it over time.
2.1.1 T Cell Activation

T lymphocytes response to microbial antigens articulates in a series of steps that result in an increased number of antigen-specific CD8+ and in T cell conversion from naive to effector state. Naive T cells are continuously generated in the thymus, and recirculate between blood and peripheral lymphoid organs, where microbial antigens are transported. The pathogenic proteins are processed and displayed by MHC molecules on the antigen-presenting cells, APCs (dendritic cells, macrophages and B lymphocytes). T cell activation by antigen and other concurrent stimuli (listed below) results in a robust IL-2 production, that induces clonal expansion. At this point, part of the T-cells differentiate from naive state (able to recognise microbial antigens) to effector state (able to eliminate microbes). A fraction of these effector cells will remain in the lymph node, while others will enter the circulation to eradicate the infection. After antigen clearance, the remaining lymphocytes will differentiate into memory T cells, which are long-lived and can rapidly respond to new exposures to the same microbes in years’ time.

Two or more TCRs and co-receptors need to be engaged simultaneously to initiate the T cell response, so the T cells need to encounter an array of peptide-MHC complexes on the APC. Also, each T cell needs to engage the antigen for at least several minutes or multiple times to generate enough biochemical signals to initiate a response. Initially integrins keep the cells in contact long enough to reach the signalling threshold. The low affinity state is then converted to a high-affinity state after exposure to chemokines, so that the cells bind strongly. B7 proteins (CD80/CD86) are over-expressed on APCs that encountered microbes and are recognised by CD28 in T cells. In absence of B7-CD28 interactions, the activation doesn’t start. Once these conditions are achieved, the T cell begins its activation program. The various interactions required for priming cytotoxic T lymphocytes are depicted in Figure 2.2, and can be summarised as follows:

- TCR recognises MHC-associated peptide antigens,
- CD8+ co-receptors recognise the MHC-class I molecules,
- adhesion molecules (integrins like LFA-1) strengthen the binding between the cells,
- receptors for co-stimulators (CD3/CD28) recognise second signals by the APCs.
Figure 2.2 Immunological synapse. This schematic represents the various bindings that occur between T cells and APCs: the TCR recognizes the p-MHC1 complex; T cells co-receptors (such as CD8) recognize the MHC1; adhesion molecules (LFA-1 and CD2) strengthen the binding around the binding site; co-receptors (such as CD3 and CD28) recognize second signals from the APC.

The biochemical pathways that link antigen recognition with T cell responses consist of the activation of enzymes, recruitment of adapter proteins and production of active transcription factors. At the beginning of the response, there is a redistribution of TCR complex, CD8 co-receptors and CD28. They coalesce to the centre and the integrins move around to form a peripheral ring. This region is called immunologic synapse. It also helps in targeting the secreted cytokines and effector molecules to the T cell. These events are of practical importance because enhancing the expression of co-stimulators may be useful for stimulating T cell responses (e.g., against tumours), and blocking co-stimulators can be a strategy for inhibiting unwanted responses.

The immunologic synapse clustering activates a protein tyrosine kinase called Lck. Lck phosphorylates tyrosine residues that become docking sites for the tyrosine kinase ZAP-70, phosphorylated itself by Lck. ZAP-70 therefore becomes active, and brings to a phosphorylation cascade near the TCR complex. There are four major signalling cascades, also schematized in Figure 2.3.

The first is the calcium-NFAT (Nuclear Factor of Activated T cell) pathway. It is initiated by ZAP-70-mediated phosphorylation and activation of the enzyme PLC\(_\gamma\), which causes PIP\(_2\) breakdown into IP\(_3\) and diacylglycerol. IP\(_3\) stimulates release of Ca\(^{2+}\) ions from the endoplasmic reticulum, raising the cytoplasmic calcium concentration. This leads to an
opening of the calcium channels and an influx of extracellular calcium into the cell, that keeps the calcium concentration elevated for hours. T-cells can be loaded with calcium-chelating fluorescent indicators to detect this increase in cytoplasmic calcium. Next, a series of reactions culminate in dephosphorylation of the transcription factor NFAT and its migration into the nucleus, where it activates promoters of several genes including the T cell growth factor IL-2.

ZAP-70 is also responsible of the *Ras/Rac-MAP kinase pathway*, that after a phosphorylation cascade leads to the final expression of the protein c-Fos and phosphorylation of the protein c-Jun. Their combination forms the transcription factor AP-1.

The third major pathway involved in T cell activation comes from the activation of *protein kinase C (PKCθ)* that activates NF-κB in the cytoplasm by breaking the complex that it forms with IκB. The released NF-κB will move to the nucleus to promote the transcription of several genes.

The last pathway, that is triggered both from TCR, CD28 and IL-2 receptors, involves the *PI-3 kinase* that leads to the activation of Akt. Akt in turn causes the activation of the protein complex mTORC1, promoter of the survival of the antigen-stimulated T cell.

All the above-mentioned transcription factors NFAT, AP-1 and NF-κB lead to an orchestrated set of transcriptional programmes that culminate in the expansion of the antigen-specific clones and differentiation of the naive T cells into effector and memory cells. Thus T cells rapidly secrete several different cytokines in the hours following the TCR:pMHC ligation, proteins that function as mediators of immunity and inflammation. CD8⁺ prodigiously proliferate in immune responses via antigen recognition and co-stimulation. Within 1-2 days after activation T lymphocytes begin *clonal expansion*, and within a week after the infection, the frequency of CTLs specific for the distinct microbial can change from $1:10^{5-6}$ to $1:5-10^7$. The fact we are interested in is that in infections with complex microbes that contain many protein antigens, a majority of the expanded clones are specific for just a few, and often less than five, immunodominant peptides of that microbe.
Figure 2.3 T cell activation pathways. The four signalling cascades of lymphocytes’ activation are schematized using different colours: calcium-NFAT pathway (yellow), Ras/Rac-MAP kinase pathway (green), PI-3 kinase pathway (red) and PKCθ pathway (purple). The stars indicate connections in the cascades (i.e. DAG production results in RAS phosphorylation, and calcium release from the internal stores triggers the extracellular uptake). These cascades promote transcription factors NFAT, AP-1 and NF-κB and protein complex mTORC1, that lead to clonal expansion of the activated CTLs and differentiation into effector or memory cells.
2.1.2 Immunodominance

APCs display many different T cell epitopes; on the other hand, each T lymphocyte clone has a unique receptor, so potentially many CD8+ can respond to the same APC. Instead, the response of one cell will dominate among the others, either in being the first cell to activate or for its long-time lasting response: the immunodominant T cell will suppress the activation of alternative CD8+ that could recognise different antigen epitopes on the surface of the same APC (schematic in Figure 2.4).

Figure 2.4 Concept of immunodominance. Lymphocytes are specific for a certain pMHC complex; in the presence of multiple antigens, a precise TCR-pMHC will prevail amongst other possible combinations, dominating the following immune response. In the representation it is shown that the red antigen, known to elicit an immune response, will be silenced in the presence of the green immunodominant epitope. Here, the hierarchical response is green > red > yellow.

Several factors determine the immunogenicity of a peptide21–25, the rate at which the APC processes the epitope, how efficiently it binds to the MHC complex and the number of pMHC presented at the surface of the infected cell are some examples. Nevertheless, the reason why some epitopes (known to activate specific T cells) don’t work when combined to other peptides has not yet been understood.

Immunodominance has been widely studied by bulk cell analysis6,26–30, but the average response of the population is not representative of the outstanding responses of some single cells. This phenomenon may be related to the first TCR:pMHC ligation (first minutes after contact), hence it is fundamental to investigate early signalling at a single cell level to highlight the response variability over the cell population. To achieve this, it is necessary to pulse APCs with the same antigen epitope, and expose single T cells to the single APC simultaneously, so that the timing profiles of single lymphocytes can be recorded in real-time (depending on the intracellular calcium levels presented by the cell, as previously mentioned) and compared.
Heterogeneity in cell biology has become a hot topic during the last decade, thanks to the development of technologies that give the chance to analyse cell populations with increasing resolution, and the first results of these studies showed surprisingly high variability even in populations of cloned cells\textsuperscript{31–36}. This heterogeneity raised the question on how reliable are the results obtained studying bulk populations of cells, since the data are averages that may mask very different behaviours and limit the chances of understanding specific biological processes related to outliers in the population. Cell diversity is logically expected among T and B lymphocytes, that need to be singularly specific to one peptide antigen among a wide range of epitopes, while not being responsive to self-cells. While CD8$^+$ immunodominance has been assessed on bulk populations, it has never been investigated at a single-cell level before. Coupled with single-cell transcriptomics, such time-resolved single-cell functional data could bring new insight to biological behaviours that have hitherto only been possible to measure as a population average. An overview of the methods that have been used up to date to study single cells is presented in section 2.2, along with the rationale behind the choice of our microwell array setup.

Immunophenotyping of single cells would be useful not only for the academic purpose of studying the immunodominance functioning, but would also give the chance to develop immunotherapies to treat cancer and infectious diseases in a way that is much more specific and less aggressive than commonly used treatments such as chemotherapy (evoking the patient’s own immune system to combat the pathogenic cells).

### 2.1.3 Cancer Immunotherapy

In cancer, it is widely believed that enhancing immunity against tumours holds much promise for treatment. Cytotoxic T lymphocytes (CTLs) represent the major mechanism by which tumour cells are destroyed, therefore the common belief is that there must be a way to elicit immune responses against specific cell types. In physiologic states, immune surveillance prevents the outgrowth of abnormal cells, but the haste of the pathogenic cells’ growth often overwhelms tumour immunity. Immunologists are interested in defining the kinds of tumour antigens against which the immune system reacts in order to enhance anti-tumour response.

While self-proteins may cause immune responses if over-expressed, tumours express antigens that are seen as non-self by the individual’s immune system. Cancer immunotherapy focuses on understanding lymphocytes activation and regulation to outline new strategies to target tumours. One idea is to vaccinate patients with their own tumour cells or with antigens from these cells. For example, it is possible to grow Dendritic cells
from individuals, expose them to killed tumour cells in vitro and use the tumour-pulsed cells as vaccines, where cross-presentation will generate CTLs against the tumour cells\textsuperscript{37–39}.

Cancer immunotherapy is nowadays moving in two main directions\textsuperscript{7,40,41}. The first one is the study of the checkpoint inhibitors of T cells\textsuperscript{42–44}, the second relies on the Adoptive Cell Transfer (ACT)\textsuperscript{45–49}.

Our immune system presents several "immunological brakes" (such as CTLA-4 and PD-1) that prevent activations of T cells in healthy conditions. Some tumours escape detection by the immune system by upregulating these checkpoint molecules that bind to the inhibitory receptors on T cells. By inhibiting T cell functions, pathogenic cells can proliferate more easily. An inhibition of these checkpoints using blocking monoclonal antibodies helps the patient’s immune system to respond more promptly and possibly find a way to recognise and target the tumour. Checkpoint inhibitors are currently in clinical use for some types of cancer: PD-1 inhibitors (Nivolumab, Pembrolizumab) are used to treat melanoma skin cancer, Hodgkin lymphoma, non small cell lung cancer and cancer of the urinary track; Ipilimumab blocks CTLA-4 and is used for advanced melanoma; Atezolizumab (PD-L1 inhibitor) is a treatment for some people with lung or urothelial cancers.

ACT is a strategy in which T cells are collected from the patient’s own blood and genetically engineered to present Chimeric Antigen Receptors (CARs) on their surface. CARs are proteins that allow the T cells to recognise a specific antigen on tumour cells. After proliferation in ex vivo cultures, CAR T cells can be infused into the patient and, thanks to their engineered receptor, recognise the cancer cells, sustaining their clonal expansion.

This project originates from the idea that if it becomes possible to distinguish the naive cells from the patient that activate more efficiently against his APCs (immunodominant cells) by studying singular TCR-APC combinations, it could become feasible to develop immunotherapies that don’t require the sophisticated extra step of genetic engineering of the patient’s cells.

A recent review\textsuperscript{50} highlights the importance of single cell (SC) studies in immunotherapies: Immune Checkpoints Inhibitors (ICI) and Adoptive Cell Transfer (ACT) proved successful in clinical trials, but need tools with SC resolution to identify the T-cell properties essential for therapeutic benefits since it is still not clear what are the characteristics and functionalities that bring T cell efficacy. TCRs does not undergo mutations after cell activation, and can be used as single cell barcode to spot clonally-related T cells. Also, having defined properties to engineer would greatly benefit ACT therapies, and obtaining TCR information at a single cell level would allow to investigate the pairing details of TCR chains. Eventually, discovering biomarkers with high accuracy and sensitivity could enable
a better patient selection into the myriad of possible trials. In the review, novel technologies to study SC proteomics and transcriptomics are listed; these techniques will be deepened in Paragraph 2.2.

2.1.4 Biological assays

Standard assays to investigate the state of activation of a lymphocyte are Flow Cytometry and Western Blot. The former allows a real-time monitoring of the intracellular calcium level of the cell population, as well as immunostaining of molecular compounds, while the latter is routinely used to determine and quantify the presence of molecules, that are indicators of the transmission or the activation signal.

Western Blot (WB) can only be used on cell lysates, and while being a powerful technique for its intrinsic resolution (e.g. to evaluate whether the molecules involved in the signalling cascade are in their basal or activated forms), it cannot be used to monitor lymphocyte activation in real time. The WB protocol can be summarized in the following steps, also represented in Figure 2.5:

- preparation of the cell lysates, to release proteins from the samples
- electrophoresis, to separate the proteins throughout the gel, according to their MW
- membrane transfer, to migrate the proteins bands onto a nitrocellulose membrane
- antibody probing, to tag the proteins of interests for detection
- imaging, to visualize and quantify the tagged bands.

Figure 2.5 Schematic of Western Blot. The lysate sample is loaded onto a polyacrylamide (PA) gel; electrophoresis will separate the proteins throughout the mesh depending on their MW (a); the protein bands will be transferred to a nitrocellulose membrane (b); the membrane will be functionalized with a primary antibody that will bind to the proteins of interest, and a labelled secondary antibody, that will bind to the primary and will be used for detection (c); the membrane will be eventually developed (d), to see and quantify the protein of interest in the various samples.
Flow cytometry is an alternative and very powerful technique that can be used to screen large populations of cells for specific characteristics and/or molecules, and more importantly allows to work with live cells, that can also be sorted when using designated devices. The working principle is schematized in Figure 2.6: the cell sample is aspirated into a fluidic circuit ending in a narrow nozzle, where cells (mostly individually) cross the pathway of an excitation laser, scattering the light depending on their specific properties. Scattered light passes through a series of optical filters, and it arrives to multiple detectors: Forward Scatter (FSC), Side Scatter (SSC) and different channels detecting fluorescence (FL1, FL2, FL3). Data is then processed through an electronic system and passed to the computer, where several software (e.g. FlowJo) can be used to visualize the events and analyse the samples. Single events (objects crossing the light in the nozzle) will appear as individual dots in the plots, and cell populations can be recognized by their size (FSC) and granularity (SSC).

It is possible to distinguish between live and dead cells, and between single cells and cell couples without labelling the sample, so that the only fluorescent tag required to monitor T cells activation over time would be on the intracellular calcium. For this reason, flow cytometry constitutes the gold standard method to be used as a reference in this project, giving an average time response of the cell line to different stimulants. The main limitation of flow cytometry however is its inability to follow individual cell activation over time. The first activators cannot be determined, as well as the different patterns in the activation signals; it is not possible to quantify the activation peaks of single cells, as these events do not necessarily occur in the instant at which the cell crosses the laser, and even more importantly there is no data on the baseline of the specific cells for a correct normalization of the fluorescence signal. All these limitations can be overcome using microfluidic tools, hence devices devoted to single cells analysis will be introduced in the following paragraph.
Figure 2.6 Schematic of flow cytometry. The sample is loaded in the fluidic system (a), and cells flow in a stream through a nozzle, that terminates into the waste line or can be collected in the case of sorting devices; when a cell crosses the excitation laser, the scattered light passes through a series of optical filters and detectors (b); forward scatter, side scatter and various fluorescence signals are acquired and passed to an electronic system, that amplifies, converts the into digital signal and forwards the data to a computer.

2.2 Single-Cell Analysis

Single-cell technologies are critical for elucidating immunological mechanisms due to intrinsic T cell heterogeneity, in fact bulk technologies make the analysis of rare subsets of cells almost impossible. In the last decade, high-throughput single-cell analysis became possible thanks to the constant development of new technologies and assays, with a wide range of targets and purposes; neuroscience and immunology are some of the fields that can benefit the most from single-cell resolution experiments.

Single-cell analysis is a wide topic that articulates at different levels, depending on the requirements of the study, and each application has its own techniques. “Omics” technologies can be divided into four main categories: genomics, transcriptomics, proteomics and metabolomics.

Genomic applications at the single cell level aim to spot genotypic differences (e.g. changes in bacterial populations, cancer evolution) that would be missed by a bulk sequencing of a sample. The starting amount of DNA will need to be amplified to allow sequencing, and current Whole Genome Amplification (WGA) strategies are “Adapter-linker PCR WGA” (controlled priming followed by PCR amplification), “Degenerate Oligonucleotide Primed – Polymerase Chain Reaction” (DOP-PCR) and “Multiple Annealing and Looping–Based Amplification Cycles” (MALBAC) (that use random priming before PCR amplification) or
“Multiple Displacement Amplification” (MDA) (where, instead of using PCR, the amplification is isothermal). Having sufficient amount of DNA, the sample is then sequenced using Sanger or Next-Generation Sequencing (NGS). Studying single-cell transcriptomics, it is possible to evaluate which genes are being expressed by the specific cell. This might be useful for example both in correlating cell characteristics and changes in gene expression (gene dynamics), or to identify cells from their expressed genes (cell typing). In order to quantify the cell transcriptome, the RNA is converted to its cDNA using reverse transcriptase. At this point, cDNA amplification and sequencing are carried out with the same techniques used for genomic studies (WTA, Whole Transcriptome Amplification). Alternatively, the transcriptome can be determined using “Fluorescence In Situ Hybridization” (FISH), where different RNA hybridization probes are employed to identify specific sequences, and can be visualized using fluorescence microscopy thanks to the attached fluorescent tags.

Single-cell proteomics aim to understand the activity of single cells, also in response to environmental stimuli, and has a similar purpose to transcriptomics. The comparison between the RNA levels (found with transcriptomics) and the protein levels (read with proteomics) gives insights in the genes that are post-transcriptionally regulated. Methods to quantify single-cell proteomics can be antibody-based (using quantum dots or fluorophores that can be visualized via fluorescence microscopy, or converting the protein levels to DNA levels, that can then be studied with genomic techniques) or mass-spectroscopy based.

Finally, single-cell metabolomics aim to investigate the rapid responses of cells to their environment through their metabolites. Metabolome cannot be amplified, hence these studies (that often focus on major biological topics such as cancer or aging) require very sensitive methods, and use techniques that include fluorescence microscopy and mass spectroscopy. Techniques based on fluorescence use proteins that can bind to the molecules of interest becoming fluorescent; mass spectroscopy is a sensitive method, and its integration with single-cell fluidics has recently extended the number of parameters that can be measured. Using rare-earth-metal isotopes, that have little signal overlap compared to fluorescent dyes, many independent cell-associated parameters, such as phosphorylated molecules, intracellular cytokines and surface proteins, can be interrogated simultaneously.

This short introduction gives an idea of the breath of current methodologies, where protocols and techniques previously used for bulk populations of cells were recently scaled down to achieve single cell resolution.

A review on the advances of single cell sequencing technologies, comprehensive of a timeline useful to realize the novelty and rapid growth of this field can be found. Amongst
the cited technologies it is possible to find protocols on WGA, DOP-PCR, MDA, MALBAC, WTA and their applications. A similar analysis can be found in 51, a recent review on single cell genome sequencing; the same protocols are in fact reported in the publication (MDA, PICOPLEX, MALBAC and DOP-PCR). Also in 54, a summary of single-cell technologies currently available is reported. Single cell qPCR, whole genome amplification technologies, molecular tagging and counting strategies are evaluated. In 2018, a new review 53, starting from the usual division of omics studies (targeting genome, transcriptome or proteome) expanded the analysis to multi-omics techniques, that combine the previous fields.

While the aim of these paragraphs is to give an idea on the information that can be extracted from isolated single cells, one of the main challenges and limitations remains the physical high-throughput screening, isolation and retrieval of the specific cells of interest, that can be downstream used for “omics” studies.

Single cell isolation has been achieved with serial dilutions, micromanipulation, laser capture microdissection, FACS, manual picking via micropipettes and Raman tweezers, but all of these techniques are relatively low-throughput, and mostly require highly-trained personnel to carry out the experiments. While being satisfactory for studies where small populations of cells should be analysed, none of these technologies has reasonable potential when the aim is to screen large numbers of single cells. Moreover, it is important to highlight that none of the mentioned methods can be used if the aim of the project is a temporal screening of thousands of single cells, to identify the cells of interest based on their peculiar signalling, or timed interaction with agonist cells. The only technology to our knowledge that is currently available to address such complex requirements, and primarily guarantee a high-throughput screening of large population of cells with potential of temporal resolution is represented by the wide field of microfluidics.

Fundamental at this point is to define the list of requirements for a platform that would allow investigation of immunodominance at single cell resolution. As previously mentioned, the goal of this project is to stimulate thousands of lymphocytes with antigen presenting cells in a time-controlled manner, with the possibility of identifying the outliers and/or to sequentially exposing the same cells to different epitopes. The design of the platform should consider the possibility of retrieval of the selected cells, that could then be further analysed using one of the above-mentioned biological assays to understand their peculiarities; alternatively, the selected cells could be put back to culture to build a population selected based on its immunological response to specific pathogens.
The experimental design must allow the following steps:

- Isolation of thousands of single lymphocytes
- Time-control of the pairing of the T cells with APCs
- Real-time imaging of cells contacts and CD8+ activation (fluorescence microscopy)
- Time series acquisition of the T cells calcium signalling and signals post-processing
- Identification of the cells of interest from their responses
- Possibility of cell retrieval and/or further on-chip analysis.

As regards the last requirement, an aspect to consider is that off-chip manipulation and “omics” analysis of the single cells will most certainly require an amplification of the DNA content (with relative noise introduction) to be able to carry on any sequencing, as previously explained. Anyhow, the use of microfluidics offers the potential to develop assays on-chip where, due to the very small liquid volumes, local analyte concentrations are high enough to directly run certain assays. This is for example the case of single cell Western Blot (scWB), a novel technology that will be further discussed in Paragraphs 2.2.3 and 7.2.1. On-chip single cell analysis becomes quite essential specifically when dealing with proteomics, since proteins cannot be directly amplified, and low quantities of molecules can only be detected by confining them in very small volumes.

### 2.2.1 Single-Cell Isolation Devices

Several reviews can be found in literature for methods to trap single cells and allow different kinds of analysis. In these reviews, different approaches for cell isolation, pairing and retrieval are mentioned, and in the next paragraph some of the most relevant publications will be highlighted. A schematic summarizing the main categories of devices for single cell manipulation will conclude the section (Table 2.1), with a summary of the reasons that drove the decision to work with microwell arrays for this project.

Since the earliest reviews of microfluidic devices targeted to study single cells, the main systems to isolate and manipulate single cells were already defined. In (2006), the devices to trap single cells were distinguished in “surface contact” and “contact-less” trapping systems: the first category included chemical, hydrodynamic traps and gel isolation, while the second grouped negative dielectrophoresis (nDEP) traps and cell manipulation using lasers, acoustic waves and magnetic fields. The same division was later adopted in (2017).

Similarly, in 2009, an updated review listed amongst the mechanisms to isolate single cells: patch clamping, hydrodynamic traps, single cell “cages” and arrays, optical tweezers,
dielectrophoretic trapping (divided in several subcategories), magnetic manipulation (via permanent magnets or electromagnets) and acoustic trapping (also this category branched off in multiple options). The mentioned publication included relevant equations for each method, making it useful for a technical understanding of the techniques.

In 2010, a new publication\textsuperscript{67} compared the cutting edge technologies for single cell analysis to the standard protocols: FACS and imaging methods (automated microscopy, laser scanning cytometry, capillary electrophoresis, laser capture microdissection). Microwells, cell patterning (via lift-off or microcontact printing), mechanical and hydrodynamic traps, magnetic devices (using immunomagnetic labelling or nanoparticles uptake), encapsulation in droplets and manipulation via optical tweezers, DEP and ultrasonic waves are reviewed. Standard protocols are also listed in \textsuperscript{68} (2015) that, while mentioning microfluidics as an emerging and promising possibility, it highlights as methods for cell isolation FACS, laser assisted microdissection, whole cell harvesting and cytosol cell harvesting using patch pipette. A different and novel method not listed before is microfluidic based on electrowetting (ElectroWetting On Dielectric, EWOD), used to manipulate droplets of cell suspension\textsuperscript{69}.

A review from 2014\textsuperscript{36} specifically focuses on single cell technologies for monitoring the immune system. It contains a powerful figure that combines the relative structure of data from single cell analyses, correlating number of cells measured, number of parameters scored and breadth of temporal resolution afforded. Microtools result to be in the favourable corner of the plot, allowing high-throughput studies and being the only technique with temporal resolution presented in the review.

In the last decade, whilst the technologies, downstream analysis and throughput of most platforms improved, the list of mechanisms used to manipulate single cells didn’t modify to the best of our knowledge. An updated analysis (2017) of the available technologies to trap single cells using hydrodynamics can be found in \textsuperscript{63}. Still distinguishing between contact-based and contactless approaches, it divides the first category in vertical and horizontal trapping methods. Vertical cell isolation comprises microwells and centrifugation-based systems, while lateral includes microfluidic chambers with traps or serpentine methods. Another review (2017) on single cell isolation techniques and methods to probe the single cells can be found in \textsuperscript{70}.

More recent literature rather focused on the emerging challenges and opportunities given by the isolation of single cells: signalling and omics analysis; these topics will be touched following. At this point, the various technologies that have been mentioned were summarized in Table 2.1, with short explanations of the working principles and a brief
evaluation of pros and cons of each method in reference to the requirements of the present study.

From the table it is possible to extrapolate that, despite the numerous techniques that can be used for single cell studies, the only ones reasonably suitable for the development of a simple, high-throughput platform to study lymphocyte signalling that could effectively be transferred to most biological laboratories would be hydrodynamic trapping, sedimentation in microwells and droplet microfluidics. A schematic of these methods is reported in Figure 2.7. Slip-chip technology is also reported in the figure (c), being a technique that has been considered and tested previously in our laboratory, and later abandoned due to the difficulty to prevent leakages and to trap and move cells in the sliding wells. Nevertheless, this method gave the idea to the first generation of a cell-pairing system that has been developed in our laboratory, and hence it is worth mentioning.
Figure 2.7 Schematics of the main techniques to isolate and study single cell responses: microfluidic arrays (Fig. a), droplet techniques (Fig. b), microwells (Fig. c) and slip-chip (Fig. d). Microfluidic arrays consist of a chamber containing traps; the first cell line is flown through the device (left to right) and cells will stick and then be pushed inside the cups; the second cell line is following flown through the chip, creating cell pairs. Droplet encapsulation of pair of cells can be achieved with a chip containing three inlets and two junctions: the first cell lines in injected from the central channel; the second will be introduced in a second inlet, that will bifurcate and connect to the central line at the first junction; the continuous phase (represented by the pattern fill) will be pumped through the third inlet, and will break the aqueous flow at the third junction, encapsulating the cell pairs into droplets. Microwell arrays are often used to isolate single cells. Up to date, cell pairing using wells has only been achieved by sedimentation of a second cell line on top of the first. Slip-chip technology consists of two plates containing wells loaded with different reagents; sliding the plates, reactions can be carried on when pairing the wells. It can be potentially applied to pair cells.
<table>
<thead>
<tr>
<th>Method</th>
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| **Hydrodynamic**       | Cells trapped/released along a microfluidic channel by creating pressure drops perpendicularly to the inlet. Microfluidic chamber containing an array of cups able to capture flowing cells. Serpentine microfluidic channel for sequential trapping of flowing cells. Cells trapped by pressure in individual chambers of a microfluidic chip. | High-throughput                                                                             Suitable for live imaging
Possibility to induce simultaneous cell-cell contact
Bulky and expensive setup (syringe pumps, fluidics)
Time consuming device fabrication
Lower throughput, difficult to scale up (e.g. risk of clogging)
Cells are in the same environment (cross-talking, no cytokine studies)
Complex cell retrieval, no post-processing on-chip                                                                                       | 71–73, 74–77, 78–80, 81 |
| **Microwell Arrays**   | Passive cell sedimentation (open system). Flow-driven cell sedimentation. Centrifuge-assisted cell sedimentation. Cell sedimentation via deformable array. Vacuum-assisted cell sedimentation. | High-throughput (possible to scale-up)                                                                                                           Easy design and fabrication of the device
Suitable for live imaging
Simple set-up, suitable for biology laboratories
Suitable for in-situ analysis (molecules contained in wells)
| **Droplets**           | Encapsulation or co-encapsulation of cells and/or beads in aqueous droplets dispersed in a continuous oil phase.                                                                                                                                                      High-throughput
Suitable for live imaging
Suitable for cell retrieval (droplets break-up)
Suitable for single cell RNA studies (no cell-cell crosstalk)
Bulky and expensive setup (syringe pumps, fluidics)
Complex design for co-encapsulation (e.g. flow rates balance)
Cell viability limited by oils and surfactants
Tendency of some reagents to partition to the oil phase
Difficult to maintain droplet stability (e.g. drops merging)
Limited choice and price of commercial surfactants                                                                                           | 128–133 |
| **Dielectrophoretic** | Electric potential that will induce a dipole inside the cells, pulling them towards electrodes covered by fibronectin | Suitable for live imaging | 134 |
| | Electric potential that will induce a dipole inside the cells, pulling them towards beads or other cells | Possible to induce simultaneous cell-cell contact | 135,136 |
| | Combination of microwell trapping and DEP manipulation | Possible to release/retrieve cells | |
| | Combination of droplet encapsulation and DEP manipulation | Lower throughput | 114,137,138 |
| | | Bulky and expensive setup (pumps/fluidics, power supply) | |
| | | Complex and time consuming device design and fabrication | |
| | | Cells are in the same environment (cross-talking) | |
| | | Not compatible with post-processing on-chip | |
| **Digital Microfluidic** | Cell encapsulation in aqueous droplets moved on an array of electrodes via electrostatic forces (Electrowetting on Dielectric, EWOD) | High-throughput | 140 |
| | | Suitable for on chip assays and droplet merging | |
| | | Complexity/price of design, fabrication, setup | |
| | | Not suitable for timed cell-cell contact | |
| **Surface modification** | Functionalization of a surface to be cytophilic (attaching adhesion promoters like laminin and fibronectin) or cytophobic (like PEG) | High throughput | 141–144 |
| | | Suitable setup for biological laboratories | |
| | | Complex fabrication of the mask/device used to create the pattern | |
| | | Limited to adherent cell lines | |
| **SlipChip** | Based on the relative movement of two plates containing wells (that will align when sliding the surfaces) – never used with live cells to our knowledge | Difficult loading of single cells | 145–147 |
| | | Potential cell damage in sliding | |
| **Optical** | IR laser beam used to move dielectric particles to the centre of the light profile (optical tweezers) | Expensive equipment and complex design | 148–153 |
| | | Lower throughput | |
| **Magnetic** | Cell manipulation via magnetophoresis (combining cells and magnetic nanoparticles) | Potential for biased results | 154,155 |
| **Acoustic** | US standing wave used to congregate cells in the antinodes | | 112,156,157 |

Table 2.1 Methods to isolate and study single cells.
In Figure 2.8, a selection of papers to show examples for each of the above-mentioned categories is reported. Figures a [158], b [156], c [81] show examples of hydrodynamic traps in which flowing cells will be trapped in small apertures of the main channel, closing the pathway for the following cells, that will be driven to the subsequent traps. While almost all microfluidic devices lack compartmentalization of the cells into isolated chambers, the example reported in figure b represents a cell pairing concept where the couples are isolated in chambers by sealing the side channels with oil. An alternative concept of microfluidic trapping arrays is represented in figures d [75], e [124] and f [144], where the cells will be trapped by posts in wider fluidic chambers that are designed to hold single cells. Encapsulation in aqueous droplets is shown in images g [159] and h [160]. Finally, isolation via open microwell devices can be observed in i [161], j [106], k [125], l [122] and m [126].

Specifically, passive sedimentation is used in i and j, while active methods to trap the cells are used in k (where cells are captured stretching an elastomeric microwell array to accommodate the cells, and releasing the device to hold the cells in position), l (where cells enter the wells via centrifugation of the device) and m (where vacuum is applied to precipitate the cells to the bottom of the wells).

An interesting comparison between microwells and microfluidic technologies can be found in 76. The authors, Burak Dura and Joel Voldman (that also developed one of the most promising microfluidic devices to trap, pair and retrieve single cells 77) shortly review published literature on immune cell interactions studied with microfluidic tools. They indicate as advantages of the microwell methods the easy fabrication and implementation of the systems in biological labs, the easier design for cells retrieval using micromanipulators, the compartmentalisation of the wells, that gives access to analysis of single cell cytokines.

Amongst the disadvantages of this technique they mention the Poisson distribution that limits the wells occupancies, meaning a low-throughput-per-footprint-area, and mostly that microwells are not suitable for dynamic studies and time controlled interactions. The present study will challenge this assumption, suggesting a novel way that has never been investigated before to our knowledge to overcome this limit, and providing 90% occupancies of the microwells. As for closed systems, they agree that the fabrication and control of the chips are more difficult, and that these technologies are more unlikely to be scaled up. Also, cells retrieval becomes more complex, and cytokines studied are limited since cells share the same fluidic chamber. Nevertheless, the main advantages are the higher throughput in cell pairing, and the time control over the interactions. They also mention that droplet methods can combine advantages from both the technologies, but that the actual occurrences of cell pairs are a very limited subgroup over the collected population, limiting the outcomes of these systems.
Figure 2.8 High-throughput methods to isolate single cells. Figures adapted from literature.
Cells isolation using hydrodynamic traps: a. illustration of the single cell trapping principle, relying on the “least flow resistance path” principle \cite{158}, b. (top image) in this case, single cell pairs are sequentially trapped using the same least flow resistance principle, and then isolated
in the individual compartments using oil [162], (bottom image) acoustofluidic chip that allows hydrodynamic trapping of single cells at a trifurcation, and to compartmentalize them in larger reservoirs via acoustic excitation [156], c. alternative designs that use hydrodynamic guiding structures to isolate single cells a microwell array [81]. Microfluidic trapping arrays: d. a dynamic single cell culture array, where cells are flown through a microfluidic chip and stopped by cup-shaped posts in a larger chamber [75], e. centrifugal microfluidic is used to trap single cardiomyocytes in the narrow gaps between arrays of posts [124], f. microfluidic sieve-like trap array used to position single cells on adhesive micropatterns [144]. Encapsulation in aqueous droplets: g. high-throughput trapping of single cells in aqueous droplets achieved evenly spacing the cells to match the frequency of drop formation [159], h. double emulsion droplets, that can be adopted to trap single cells and promptly screen them using flow cytometry [160]. Isolation in open microwell devices: i. Polymer Live Cell Array, also used to co-incubate T cells and APCs within the same well [161], j. agarose microwell array used to run comet assays on-chip on single cells [106], k. loading of single cells in a PDMS microwell array by stretching and releasing the elastomeric chip [125], l. centrifugation-assisted single cells trapping in microwell arrays [122] and m. cells isolation in a silicone microwell array with pores on the bottom of the wells, achieved applying a negative pressure under the chip [126].

Given the increasing popularity of single cell technologies, several instruments for single cell collection and analysis have been commercialized. A recent review163 (2018) includes an overview of the devices currently on the market. All instruments have been commercialized within the last 5 years, and the vast majority relies on droplets or microwells technologies. Reviewers indicate that microwells devices are lower throughput compared to droplets platforms, but allow visual inspection of the cells sample. Microwell platform are also indicated as more flexible to adapt to own protocols.

A market survey (2015) on technologies for single cell isolation164 was also conducted to identify the most relevant methods routinely used by scientists. According to this study, researchers looking for single cell isolation methods prevalently used FACS (33%), followed by laser microdissection and manual cell picking (17%) and random seeding/dilutions (15%). Microfluidic devices were used by 12% of the surveyed scientific community, that overall showed an increasing interest in single cell isolation tools: 179 patents were identified on emerging technologies addressing the need for resolution from biologists. As for the researched topics, immunology and oncology proved to be the far prevalent fields. These data give a hint on the importance and growing interest on single cell studies, and the newly developed protocols to analyse single cell omics feed the need for high-throughput yet simple platforms that can perform the initial screening on large population of cells in biology laboratories.
2.2.2 Single Cell Temporal Resolution (cell-cell contact)

A growing number of reviews investigate the literature on cell-cell interactions studied with microfluidics. Most of the devices rely on the mechanisms illustrated in the previous paragraph. These devices have been optimized in order to accommodate a second cell line in the trapping system, paving the way to a new set of analysis on signalling events. As already mentioned, the time resolution is a brand new characteristic offered by microfluidic tools, hence the access to such mechanisms became appealing.

A recent (2018) review screened the reported devices based on their applications, both in single cell studies (for cancer biology, immunotherapy and neurobiology) and in cell-cell communications (used to investigate cancer biology/immunology/therapy, neurobiology and vascular models). As for single cell studies with the aim of assessing immunotherapy, the reviewers focused on two devices that aimed to develop cancer vaccines: in the first paper, dendritic cells and tumor cells were hydrodynamically trapped and electrofused using positive dielectrophoresis (pDEP), then released using negative dielectrophoresis (nDEP); the second paper refers to a single-cell high-throughput chip for cell transfection, where cells are temporarily perforated to take up molecules.

Cell-cell communication studies are also reviewed in (2017), where the devices are divided based on the kind of interaction that they aim to analyse: long distance communications (synapse, migration and co-culture devices) and short distance signalling, involving vertical and horizontal cell pairing. In both cases, the involved technologies are grouped in: structure traps, microwells, droplets, electrode-based and acoustofluidic devices. When comparing vertical and lateral cell pairing systems, the reviewers highlight as advantages of the vertical alignment the better resolution on the cell-cell interface thanks to confocal microscopy and the possibility of acquiring Z-stacks of the interface. As for the disadvantages, they report the more difficult multilayer fabrication. Specifically, when describing immune cell heterogeneity studies, two relevant papers are mentioned in the review, were microwells were used to pair T cells and APCs: (where APCs and T-cells were sequentially loaded into a microwell array with high micropits) and (where a microfluidic device with two layers of traps could host the cell pairs vertically).

One last review to mention was published in 2014, and it analyses microfluidic platforms that investigate various cell signalling mechanisms: gap-junction signals, contact-dependent (juxtacrine) signals, autocrine signals, paracrine signals and synaptic signals. As for juxtacrine signalling, the reviews refer to micropatterning techniques (bowtie structures where to adhere cell pairs), droplet-based fluidics, Bio-Flip Chip to isolate and pattern single...
cells on a substrate flipping a microwell array on a dish\textsuperscript{170} and to a T cell study\textsuperscript{171} that uses an ancestors’ system of hydrodynamic traps to the one developed by Voldman’s group.

As for the single cell isolation systems, the main devices that have been used to pair single cells are reported in Figure 2.9.

The first group of devices represents solutions that have been applied to hydrodynamic traps to couple cells (figures a \textsuperscript{71}, b \textsuperscript{172}, c \textsuperscript{79}, d \textsuperscript{173}, e \textsuperscript{77}). Specifically, a \textsuperscript{71} and b \textsuperscript{172} are very similar devices design to initially trap the first cell line applying a vacuum force to one side of the main channel, and repeat the process with the second cell line, applying the vacuum to the other side of the line. Having apertures on the sides of the channels that are aligned, it is possible to create several cell pairs. Similarly, in c \textsuperscript{79}, the first cell line is flown in a main serpentine channel and individual cells are sequentially trapped through apertures along the line; by flowing the second cell line in the opposite direction once the first cell line has adhered, it is possible to create cell pairs in a sequential manner. Figure d \textsuperscript{173} has been included although it refers to single cell trapping and subsequent release in individual chambers of a microfluidic chip since, by repeating the process with a second cell line after the first has settled, it should be possible to incubate cell pairs in larger chambers. Eventually, figure e \textsuperscript{77} refers to the most promising technique to create high numbers of cell pairs in U-shaped traps within the same fluidic chamber. The Voldman group showed how they improved the technique presented in \textsuperscript{75} in order to synchronise the cell pairing across the array and to isolate cells of interests afterword, using a micromanipulator (refer to following session).

Following, a selection of papers purposing cell pairs contact induced with dielectrophoresis (DEP) is shown in figures f \textsuperscript{137}, g \textsuperscript{114} and h \textsuperscript{138}. All three systems work in combination with a form of physical constraint to the cells (traps or wells). Briefly, in all cases cells are locked into specific positions by activating electrodes. In figure g, the cell pairing occurs vertically, while in the other two instances, the second cell is trapped next to the first with a different set of electrodes. The cells are then paired either applying hydrodynamic pressure\textsuperscript{137} or pushing the cells with negative-DEP\textsuperscript{138}.

Cells co-encapsulation in droplets is represented in figures i \textsuperscript{129}, j \textsuperscript{139} and k \textsuperscript{174}. The device pictured in figure i represents a complex cell pairing mechanism, where two cells are co-encapsulated by merging of different droplets via electrocoalescence; the droplets are then shrunk (75% volume reduction) using auxiliary channels, where satellite droplets are generated, allowing cell-cell membrane contact; ultimately, hybridoma are formed by electrofusion of the two cells. A simpler yet effective design for cells co-encapsulation in presented in \textsuperscript{175}, where early activation of T cells was studied. Single human T cells were co-
encapsulated with dendritic cells activated by ovalbumin peptide, and dynamic calcium signal monitoring followed (proving a marked heterogeneity over the population). Delayed responses were also recorded among T cells co-encapsulated with pulsed DC but not in contact; this recalls the drawback of such platform, that doesn’t give any possibility of control on the timing and/or occurrence of the interactions. The working principle of the device in figure j is slightly different: droplets containing cells are sorted to discard the numerous empty droplets; the two samples (beads in this case) are encapsulated in droplets of different sizes. The droplets are later trapped in an array of paired microwells, design to contain one big and one small droplet only; flowing a demulsifier it is possible to cause drag force-driven physical contact between two paired droplets, triggering their merging. The merged droplets can be retrieved from the device by flipping the chip. As regards figure k, it refers to a 2018 paper that investigated TCR T cell therapy, hence a very relevant and valid alternative platform to the one introduced in the present study. T cells and antigen presenting cells are co-encapsulated in droplets, that are then trapped in a microwell array to allow monitoring of the specific cell pairs. Differently from the suggested method and from Voldman trapping array, the timing of the cell-cell contact cannot be controlled; although the long times of observation, this discrepancy might influence the relevant cells selection and downstream analysis. Nevertheless, the paper introduces an interesting technique to retrieve specific cell pairs: the group used a UV-laser microdissection microscope to heat-induce cavitation of the oil under the selected droplet in the well. The formed bubble would push the droplet out of the well, that could then be dragged to a recovery chamber flushing fresh oil through the device.

Figures l [176], m [123] and n [177] refer to microwell devices. As mentioned in the previous paragraph, one of the main drawbacks attributed to microwell systems was the fact that they are not directly suitable for cell pairing in a time controlled manner. As a matter of fact, each paper addressed this challenge differently. Figure l shows a device composed of two well-plates (a capture microarray having wells with diameter:height 25:30µm and a culture microarray with traps of diameter:height 285:300µm) that are face-opposed and enclosed in a microfluidic system. Cells are loaded in the trapping plate via syringe pump and passive sedimentation, and then the device is flipped and single cells fall in the facing culture wells. While being a device for single cell isolation and culture, it could be used for cell coupling by adhering the first cell population on the bottom of the culture wells as an initial step. There is literature178 supporting the idea of cell growth and stimulation inside microfluidic channels. Nevertheless, with this setup it would be difficult to define a synchronous time-zero in the cell-cell contact. Figure m is the upgrade of the platform described in Figure 2.8, l [122]; cells are paired by subsequent centrifugation in the same array wells. Figure n, instead, suggests another new technique based on a stretchable PDMS device, where deformable L-shaped microwell arrays are pulled in one direction to capture the first cell population, and
next in the perpendicular direction to trap the second cells sample. Cells will be hence in communication through the angle of the shared well.

The last device (shown in figure o [179]) introduces a new (2018) concept of cell pairing, suggesting a way to pneumatically drive the cell-cell interaction: cells are adhered onto pillars that lay on top of a fluidic channel. Pressurizing the channels, the pillars will be bent apart from each other, while when the pressure is released, the two cells will come into contact. A similar idea drove the design of a microfluidic platform to pair T cells and APCs in this project, and it will be introduced in Paragraph 6.3.
Figure 2.9 Cells pairing devices. Review of technologies that can be used for single cell signalling studies, images adapted from literature. Cell coupling using hydrodynamic forces: a, b, cells are passed through a chip while a negative pressure will drag them to block small pores on one side of the channel; sequentially and symmetrically, the second cell line will be trapped on the other side of the channel, creating cell pairs [71,172], c, cellular valving principle used to create cells couples: the first cell line is trapped following the least flow resistance path, hence it adheres and flattens to the bottom of the channel, opening the aperture again; the second
cell line can be trapped similarly to the first by flowing the chip in the opposite direction [79], d. cells are trapped sequentially following the least flow resistance path, hence moved into individual large reservoirs by inverting the flow [173], e. cells are trapped in U-shaped cups, hence pushed inside the cup increasing the fluidic pressure; the same process is repeated for a second cell line, creating simultaneous cell-cell contacts [77]. Contact induced with dielectrophoresis (DEP): f. planar DEP-based chip where couples of single cells are trapped by sequentially activating arrays of electrodes; once combined, the cells are released and the pairs pushed into collecting cups by hydrodynamic forces, creating cell-cell contact [173], g. cells are paired vertically using positive-DEP: cells flowing in the chip are attracted into the wells by the active electrode, and retained during cells washes; the electrode is activated a second time to trap the second cell line [114], h. the two cell lines are sequentially trapped into facing microwells using positive-DEP; hence they are pushed into contact using negative-DEP [138]. Co-encapsulation of two cells in one droplet: i. the device articulates in a double T junction to automatically pair cell couples, three pitchforks to shrink the droplets and an electrode pairs to induce cell pairs fusion (electrocoalescence) [129], j. the chip allows to generate droplets containing single cells, sort the cell-positive droplets and trap them in a microwell array; the process can be repeated with droplets of a different size, creating droplets pairs in the array, then merged using a demulsifier. [139], k. the device allows co-capturing of a TCR T cell and a target cell droplet having a double aqueous feed; droplets are then trapped in an array to allow imaging [174]. Dual well device, that could be adapted for cell pairing: l. cells are singularly trapped in small wells, then moved to larger wells by flipping the chip [176]. Centrifugation-assisted cell pairing in microwell array: m. two cell populations are trapped in vertical pairs in microwells following two centrifugation steps [122]. Cell-cell coupling via stretchable L-shaped wells: n. cells are sequentially trapped in L-shaped microwells by stretching the chip in alternate directions in order to trap and retain the cells [177]. Pneumatically-driven cell-cell interaction: o. two myocytes are adhered onto pairs of posts that live on top of a microfluidic channel; when the channel is pressurized, the posts (and cells) won’t touch, while contact will be created when a negative pressure is applied in the fluidic system [179].

2.2.3 Subsequent analysis and cell retrieval

As mentioned in [56], “A single cell weighs 3–4 ng and has a volume of ∼1 pL and a diameter of ∼10 μm, depending on the cell type. The total protein content of a cell is ∼700 pg, but the dynamic range of concentration can span up to seven orders of magnitude. The mass of genomic DNA is estimated to be ∼6 pg, whereas that of RNA is ∼10–20 pg. Therefore, it is technically challenging to resolve single-cell events by using conventional detection techniques, which require micrograms of sample components”. These numbers give an idea on the reason why microfluidic tools are becoming so fundamental in single cell analysis.

The review screens the types of devices that can be used (microwells, surface patterning, hydrodynamic traps, droplets, SlipChip technology and micropillars) to then focus on applications of these devices: PCR-based analysis, genomic and transcriptomic studies, cytogenetic analysis using FISH, study of intracellular proteins, secretory profiling and metabolites.

Some of the uses of these devices are also listed in other reviews. In [180], for example, PCR activated cell sorting in microdroplets, single cell western blotting and motion and growth of circulating tumor cells in microwells are mentioned. [181] is a review on single cell genetic analysis using microfluidics. Possibilities of applications to drug discovery (e.g. droplet-
based cytotoxicity screening) are mentioned in 182 and in 69. A short review on single cell RNA sequencing can be found in 183. The recent combination of microwell and microfluidic technologies in a device that allows microwell-based single cell RNA sequencing are also reported in a research highlight from 2015 184. The different approaches when it comes to short or long term monitoring of single cells (mostly PCR on-chip approaches VS long term incubations) are described in 185.

A review186 from 2018 studied single cell isolation and post processing technologies. Integrated valve traps (Quake valves, from their inventor) allowed Multiple Displacement Amplification (MDA), digital PCR, whole-transcriptome and protein analysis. Droplet technologies gave the possibility to cultivate, merge cells via electroporation, perform antibodies, enzyme and drugs screening, run single cell PCR and RNA sequencing (RNA-seq), whole-genome amplification, and chromatin immunoprecipitation for sequencing (ChIP-seq).

As mentioned earlier in the chapter, while genomic and transcriptomic material can be amplified with PCR-based methods, this cannot be achieved when it comes to proteomics. A review on single-cell proteomics can be found in 34 (2017). Some of the ideas researchers came out with to study such small quantities are listed: photocleavable DNA labels that can be attached to antibodies to enhance multiplexing, the Single Cell Barcode Chip (SCBC, a device containing a barcode pattern to help capturing low numbers of target cells), a microwells platform enclosed by an immunoassay lid, capillary electrophoresis, single cell western blot (also the updated version to enhance the gel resolution), the combination of cells and cytokine-capture beads in droplets (also using agarose-based droplets to be able to wash-off the unbound cytokines and higher the specificity).

While keeping an eye on possibilities opened by the choice of the single cell isolation method, the present study aimed to study single lymphocytes signalling in a platform that could then be enhanced to allow further analysis on the identified cells of interest. For this reason, the various omics analysis that could be later performed will not be investigated further. Nevertheless, what is important at this stage of the project is to evaluate the directions that could be taken after signalling studies: either on-chip analysis or single cell retrieval.

In Figure 2.10, a selection of cell retrieval methods is shown.

Following the Drop-seq technology launched by McCarroll’s group, Sims’s group developed a microwell-based single cell RNA-Seq: cells are enclosed in the wells flowing oil in the chip, and their lysates are individually captured on barcoded beads, later rescued from the device; its working principle is represented in image a [115].
In figure b, a pressure-driven single cells sequential retrieval from microfluidic chip is presented. The image was obtained from single frames of an explanatory video that can be found in the Supplementary Information of the publication to simplify the working principle to the reader. The device has been used to study lineages of single cells.

Figure c, taken from a review paper, sums up the single cell isolation protocols based on Laser Capture Microdissection (LCM); this method is routinely used on dissection of living tissue, but has potential to be transferred on chip. The first example is “contact-based extraction”, achieved cutting around the cell with a laser and retrieving the sample with adhesive tube caps; the second example shows the “contact-free gravity assisted microdissection”, where the substrate faces down, onto a collector tube; the last image refers to the “contact-free pressure catapulting” technique, where the defocused laser pulse is used to generate a local plasma under the cut cell, that moves the cell against gravity to a collector container.

The technology presented in figure d originally come from Allbritton’s group, and later gave birth to a startup company (CELL Microsystems), that commercialized the platform in few variations. Cells are singularly adhered onto an array of micro-rafts, that can be individually released puncturing the elastomeric membrane underneath with an automatized needle mounted on the microscope objective; the raft can then be magnetically captured and transferred to 96-well plates for downstream analysis.

Figure e shows how cell couples have been retrieved from the Voldman’s microfluidic chip described before. Cells are locked in position by flowing a solution of agarose, that can be solidified lowering the temperature of the system; hence a micromanipulator is used to puncture and sequentially retrieve the cell pairs of interest.

In figures f and g, retrieval of single cells/barcoded beads using photopolymerization is shown. The first publication uses a dual-photopolymerisation procedure to encapsulate undesired cells and retrieve the selected ones with a washing step. The second paper is based on Stochastic Particle Barcoding; single cells and a random collection of fluorescent beads are co-encapsulated in enzymatically-degradable hydrogel blocks; the number, colour and position of the beads define the specific barcode of the cell, so that samples can be transferred in bulk to other platform keeping track of the single cell identity. Codes can then be read using a fluorescence microscope, and the hydrogel can be depolymerized, freeing the cells of interest.
As previously stated, all these methods originate by the limit of microfluidic platform of performing an on-chip analysis. Where on-chip analysis is possible, it is preferable since it means cutting the time consuming step of retrieving the cells of interests, and the amount of reagents required to perform downstream assays. Microfluidic devices that aimed to perform
bodies protocols on-chip are usually very complex in the design, fabrication and control, being mostly based on multilayer valve structures to compartmentalise determined areas of the chip. PDMS devices are usually disposable, and complex and long preparation of the chips means intrinsically low-throughput studies. Likely the best example to date of on-chip analysis that can be achieved using microwells technology is represented by the single cell western blot \cite{92,191}, here described. As mentioned in the publications, this detection method is quantitative, multiplexed and at-the-bench operated; it can be integrated to upstream fluorescence microscopy screening of the cell population, allowing correlation of data in a high-throughput manner. The platform consists of a microwell array made of a photoactive polyacrylamide (PA). After cell loading and observation via microscopy, cell lysis is induced adding RIPA buffer, that solubilizes the intracellular proteins; electrophoresis is immediately initiated, and proteins can then be photo-immobilized to the PA sheet thanks to benzophenone moieties added to the matrix of the gel. Hence the slide can be probed, stripped and reprobed multiple times. Imaging can be achieved with a fluorescence microarray scanner. Single cell western blot (scWB) technique enables the targeting of molecules despite their location within the cell and without cell. Because of the difficulty of measuring intracellular and signalling proteins (e.g., phosphorylated proteins) with FACS and surface methods, the microwell platform that will be introduced in this dissertation could be a great candidate for an integration with scWB downstream the timing-competition analysis.

### 2.2.4 Immunology-on-a-chip

A very recent review\cite{59} (2018) focused on microfluidic single-cell technologies used in immunology and antibody screening. The paper touches on themes like T cell activation signalling and cell-cell interactions, immune cell migration and phenotypic screens for antibodies with desired properties. Ultimately, it analyses the screening of B/T cells repertoires. For each session, microfluidic devices used to assess the various topics are divided into microwells, continuous flow, valve-based and droplet-based. As for the T cell activation and cell-cell communications, several examples that highlight heterogeneity within the same immune cell type are reported. All of these could not be spotted with standard techniques (cytokines capture with antibodies immobilized onto a membrane and flow cytometry). As reported, since the T cell activation occurs within seconds, real-time imaging becomes important. Love’s platform to capture molecules secreted by T cells isolated in wells using an antibody-functionalized glass lid\cite{192} is mentioned (a similar approach can be found here\cite{116}). As for droplet-based technology, cited devices include Konry’s\cite{175} and Chokkalinga’s\cite{31} platforms, where cytokines are captured on the surface of
beads co-encapsulated in the droplets. A more complex platform involving valve-based fluidics is reported\(^3\). It is worth mentioning Lu’s platform\(^{2015}\), where 45 proteins could be detected in one assay (similar to Love’s approach). Hence, Dura’s platforms\(^{189,193}\) are described both for the cytokines analysis and for the cell-cell interaction studies. As for the cell-cell interaction, cell centrifugation to induce contact was used in the past\(^{194}\), and alternatively to Dura’s fluidic platform it has been tried with droplets by Sarkar’s group\(^{175,195}\). As for the screening of T cell repertoires, TCR heterogeneity and pairing characteristics should be studied to help with two major immunotherapies: infusion of autologous antigen-reactive T cells expanded in vitro and infusion of de-novo generated antigen-reactive T cells produced introducing genes encoding antigen-reactive TCRs. Georgiou’s lab used droplets to implement a high-throughput sequencing of TCRs\(^{196}\), and Teichmann’s lab developed the TraCeR package, to correlate single T cells’ TCR and transcriptome obtained by Fluidigm C1 systems. Similarly, single cells RNA-Seq methods such as DropSeq\(^{197}\) and InDrop\(^{198}\) can be used to extract even more data on the specific cells.
Chapter 3: Material and Methods

In this Chapter, biological standard protocols that have been used throughout the project will be explained. Cell lines choice and culture technique, the maintenance of lymphocytes’ sensitivity via selection of responsive subclones, cell staining and flow cytometry analysis protocols will be detailed.

3.1 Cells Selection and Maintenance

Murine CD8+ T cell hybridoma B3Z has a TCR specific to antigenic peptides derived from ovalbumin (OVA), like SIINFEKL (SL8) and SIINFEHL (SHL8); these peptides can be presented by K\textsuperscript{b} MHC-I molecules on the surface of antigen presenting cells. K89 is a mouse L-cell line expressing the relevant H-2K\textsuperscript{b} molecule, and can be used for exogenous peptide stimulation assays\textsuperscript{199-202}. For this reason, B3Z and K89 were selected as a model system.

All the cell lines used in this project have been previously cultured by our group, unless otherwise indicated, and aliquots were thawed from liquid nitrogen when needed in order to normalise the passage number across experiments. Cryovials were warmed up in a 37°C water bath, and when the content was defrosted, the cell solution was pipetted into a falcon containing a pre-heated 1:1 mixture of fetal bovine serum (FBS) and growth media. The freezing mix was removed by washing the cells twice (290 g, 5 min, room temperature) in fresh culture media, and cells were then moved to a flask to culture.

RPMI 1640 culture medium (with L-Glutamine, Gibco, 21875-034) was supplemented with 10 %v/v heat-inactivated FBS (standard quality, EU approved, GE Healthcare, A15-101), 1 %v/v Penicillin-Streptomycin (Sigma, P4333), 1 %v/v HEPES Buffer (1 M, Lonza, BE17-737E), 1 %v/v Sodium pyruvate solution (100 mM, Sigma, S8636) and 500 nM β2-Mercaptoethanol (Sigma, M3148).

Adherent K89 and suspension B3Z were cultured in complete RPMI at 37°C, 5 % CO\textsubscript{2} and harvested every two days (or as otherwise required). 1 mM EDTA (Biowhittaker, BE17-711E) was used in the passaging. After removing the growth media, 4 ml of solution were added to the flasks, that were then incubated at 37°C, 5 % CO\textsubscript{2} for 5 minutes. Cells were gently detached from the flasks mechanically, using a pasteur pipette, and the cell suspension was added to a falcon tube containing 10ml of pre-warmed culture medium. After spinning down the cells (290 g, 5 min, room temperature), the pellet was dissolved in fresh culture media, and resuspended 1:10 in 10 ml of complete growth media to keep the culture.
Before the experiments, cells were counted and the required amount was resuspended at the necessary concentration. To do so, 10 µl of the cell suspension was mixed with the same amount of trypan blue solution (0.4 %, Gibco, 15250-061), and live cells were counted either manually using an haemocytometer or with LUNA Automated Cell Counter.

Alternative APCs have also been investigated as part of the study, in order to confirm that the B3Z-K89 combination was the best for our model, and to investigate the trapping efficiency of different cell lines in our microwells platform. Specifically, the following cell lines (presenting Kb complexes) have been tested and cultured using the same protocols as for K89 and B3Z:

- EL4, suspension murine lymphoblastoma;
- FS-DC, semi-adherent murine dendritic cells;
- B16, adherent murine melanoma cells.

All cell lines were maintained in complete RPMI growth media, according to the same protocols above depicted.

In experiments reported in 6.2.4, HeLa cells (adherent human cervix adenocarcinoma) have been employed. These were kindly provided by Marios Stavrou (West’s group, University of Southampton, UK) and cultured in DMEM (Gibco, 11995-065) supplemented with 10 %v/v heat-inactivated FBS (standard quality, EU approved, GE Healthcare, A15-101) and 1 %v/v Penicillin-Streptomycin (Sigma, P4333), according to the above described protocols.

Cell stocks were frozen down to create a backup of each cell line. To do so, 2*10⁶ cells were resuspended in 500 µl of full media in each cryovial, and when all samples were ready, 500 µl of freezing mix (1:4 parts of DMSO:FBS) were added to each and mixed. Samples were transferred to a -80°C fridge in a Mister Frosty and subsequently moved to liquid nitrogen, or alternatively transferred to the gas phase of the nitrogen tank and moved to the liquid nitrogen following.

### 3.2 Dilution cloning to enhance population sensitivity

B3Z T cell hybridoma was initially subcloned to obtain populations with higher sensitivity to SIINFEKL (SL8) bound to H-2Kb presented at the K89 cell surface. Sensitive subclones were frozen down to use throughout the project. Sensitivity of the population in culture was anyway tested periodically, to guarantee that the cell model was correctly functioning when testing the engineering platform.
In order to start the subcloning, cells of the original B3Z population were counted and sequentially resuspended to a statistical concentration of 5 cells/ml. 200 µl of the final cell suspension were added to each of the 96-wells in two flat bottomed cell culture plates, theoretically giving a single cell per each well. B3Z subclones were cultured for 9 days, periodically replating in bigger wells to maintain the exponential growth. At day 7, wells positive for B3Z cell growth were harvested and tested for sensitivity to SHL8 or SL8 peptides.

B3Z expresses the *Escherichia coli* lacZ reporter gene for β-galactosidase in response to low concentrations of SL8 (<1 pM) bound to the H-2Kb complexes of L-cells. To distinguish B3Z subpopulations specific to the peptide, for each subclone 100 µl of cell suspension containing 10^5 cells were added to two different 96-well plates. K89 were harvested and resuspended to the same concentration as B3Z, and divided in two tubes. One of the two would contain plain media, while 10 nM SHL8 were added to the other. K89 were added at a 1:1 K89:B3Z ratio to a 96-wells plate, and K89-SHL8 were added at the same ratio to the other 96-wells plate. Cells were co-incubated overnight at 37°C, 5 % CO₂. Cells were then pelleted (290 g, 5 min, room temperature) and the supernatant was removed and replaced with 100 µl of a mixture of CPRG (CPRG; 91 mg CPRG, Roche), 1.25 ml Nonidet-p40 (Sigma) and 9 ml 1 M MgCl₂ (Sigma) per 1 litre phosphate buffered saline (PBS) for each well. CPRG is a chemiluminescent substrate that is cleaved in the presence of β-galactosidase, resulting in a colour change in the presence of lacZ activity of B3Z cells.

The plates were left to react at room temperature, and the colour change was quantified after 6 hours using a BioRad 680 microplate reader. Readings were taken at a wavelength of 595 nm and at 695 nm as a reference. Results are depicted in Figure 3.1, illustrating that the wells that were positive to cell growth were all specific to the presentation of SHL8 at the K89 surface, showing lacZ activity only in the presence of peptide. This confirmed that the population could be used as a cell model for the experiments. The top 12 responders were selected, and the corresponding subclones were kept growing in 1 ml media in a 12-wells plate. After 5 days, the clones were tested for their sensitivity (see 3.2.1), and upon confirmation of the good quality of the cultures, they were resuspended in 2 ml in 6-well plates. A new sensitivity assay was run after other 4 days of culture, and the best population was cultured to create a great stock of sensitive B3Z, to be used throughout the project. It is important to stress out that most of the experiments ran on the microwells platform were performed using much higher concentrations of peptide (1 µM if not otherwise specified), since the primary purpose was to have a valid cell model to be used in the optimization of the device (i.e., highest possible responses). In any case, to ensure a good performance of the cell line over time, new cryovials were periodically defrosted and tested for sensitivity.
Figure 3.1 Results from the specificity test ran on two 96-wells plates of the B3Z subclones. Each subpopulation from a single well is depicted by a histogram column in the top diagram and the correspondent column in the bottom graph; left and right plots represent the two 96-wells plates. Absorbance was measured with a microplate reader at a wavelength of 595 nm. The top graphs represent the lacZ activity of subclones in response to K89 loaded with SHL8 peptide, while the bottom graphs represent the lacZ activity of subclones in response to K89 alone (control). Populations in red were selected for culture and subsequent testing.
3.2.1 T cell sensitivity assay

Sensitivity assays on larger subclones and newly defrosted populations of B3Z (where the number of cells were high enough to allow it) were ran using different peptide concentrations, to actually measure the limit of detection for the specific culture.

B3Z (from each clone) and K89 were counted to decide the extent of the titration; usually, 4 to 7 peptide concentrations were tested on each sample, and for each well $10^5$ B3Z were required. 100 µl of cell suspension containing $10^5$ K89 were added to all the wells of the assay in a flat-bottomed 96-well plate; extra 100 µl of the suspension were added to the first column of the assay. SHL8 or SL8 peptides were added at the initial concentration of 10 pM to the K89 cells in the first column, and they were titrated down for each row of the plate, diluting the peptide 1:2 at each step. No peptide was added to the last column, as a control for the specificity of the samples. At this point, for each B3Z clone, 100 µl of cell suspension containing $10^5$ cells were added to each well of the corresponding row. A schematic of the assay is depicted below.

As for the specificity test, cells were co-incubated overnight at 37°C, 5 % CO₂. Cells were then pelleted (290 g, 5 min, room temperature) and the supernatant was removed and replaced with 100 µl of a mixture of CPRG (CPRG; 91 mg CPRG, Roche), 1.25 ml Nonidet-p40 (Sigma) and 9 ml 1M MgCl₂ (Sigma) per 1 litre PBS for each well. The plates were left to react at room temperature, and the colour change was quantified after 6 hours using a BioRad 680 microplate reader. Readings were taken at a wavelength of 595 nm and at 695 nm as a reference. In Figure 3.2 an example of the outcome of a sensitivity assay is reported. Specifically, results for 5 of the cultured subclones are presented and compared to the sensitivity of the starting B3Z population (control), showing that the dilution subcloning
allowed to improve the sensitivity of the cells used for the model system. Subclone F6 (sub F6) was chosen to create a B3Z stock to use throughout the project.

![Figure 3.2 Sensitivity assay of the best subclones. LacZ activity of B3Z subclones is quantified in response to various peptide concentrations loaded on the K89 APCs. The control represents the starting population from which subclones were selected. It is shown how the dilution cloning method allowed to improve the sensitivity of the model system. The name of the subclones refers to the initial well of the 96-well plates in which the various populations grew. Sub F6 was selected to create B3Z stocks. Similar assays have been run throughout the project to control the sensitivity of the culture.](image)

### 3.3 Staining Protocols

In order to monitor the calcium fluctuations of T cells over time, prior to each experiment the lymphocytes were stained using Fluo-8AM (ab142773, Abcam). This medium affinity green fluorescent calcium indicator (excitation 490 nm, emission 520 nm, Kd = 390 nM) can permeate the cell membrane being hydrophobic and uncharged, and once in the cell cytoplasm, hydrolysis carried by intracellular esterases prevents the molecule from leaving the cell. The dye binds the free calcium in the cytoplasm, and it is suitable both for flow cytometry and fluorescence microscopy. Due to the basal intracellular calcium level, the stained cell population will always have a basal level of fluorescence (measured as Mean Fluorescence Intensity, MFI), and fluctuations in the ions cytoplasmic concentration will result in an increased/decreased fluorescent signal.

B3Z T cells were resuspended at $10^6$ cells/ml in growth medium (or as otherwise stated for the specific experiment), and Fluo-8AM was added to a final 5 µM. The samples were incubated in the dark at 37°C, 5 % CO₂ for 30 minutes, followed by a further 30-minutes incubation at room
temperature. Cells were then centrifuged (290 g, 5 minutes) and resuspended in fresh culture media; this washing step was repeated 3 times. As shown in Figure 3.3, 5 µM Fluo8-AM concentration was sufficient to capture the highest calcium oscillations (subsequent to stimulation with the calcium ionophore ionomycin), while a lower concentration would have been unsuitable for the study, giving poor data quality.

Figure 3.3 MFI of B3Z populations stained with different concentrations of Fluo8-AM (2.5µM, 5µM and 10µM). Each number on the x-axis represents a different sample. For each sample, the basal MFI was recorded using flow cytometry; following, the increase in fluorescence due to a stimulation with 20 µg/ml or 100 µg/ml antiCD3 was documented; eventually the sample was pulsed with 1 µg/ml ionomycin, giving the highest MFI. 10 µM and 5 µM dye concentrations gave similar readings, while 2.5 µM gave low signals. As such, 5 µM became the Fluo8-AM concentration of choice.

In experiments where APC were used, the K89 cell population was stained using a red membrane dye (PKH26, PKH26GL, Sigma-Aldrich; excitation 551 nm, emission 567 nm) or the Orange CMRA CellTracker (C34551, ThermoFisher; excitation 548 nm, emission 576 nm). Staining both cell populations helped in the initial gating using flow cytometry and to confirm the relative localization of single T cells and APCs using the custom rig under the microscope. The partial overlap of these dyes with the fluorescence spectra of Fluo8-AM (Figure 3.4) did not interfere significantly with the data analysis under the used conditions.

When staining with PKH26 dye, K89 were detached and 10⁶ cells were resuspended in 250 µl of Diluent C. 250 µl of PKH26 4 µM (obtained diluting the 1 mM stock in Diluent C) were added to the cell suspension. The sample was left for 3.5 minutes shaking at room temperature, then 500 µl of FCS were added. After another minute at room temperature, 1 ml of fresh media was added. The sample was then washed twice (290 g, 5 minutes), resuspending the pellet in fresh PBS between the washings.
When using Orange CMRA CellTracker, a 1 µM dye solution in serum-free medium was prepared from the stock (10 mM CellTracker in DMSO) and warmed up to 37°C. Growth medium was removed from the K89 culture plate, and the dye solution added. Cells were incubated at 37°C, 5 % CO2 for 30 minutes, then the dye solution was removed and upon addition of fresh culture media, cells were ready for imaging.

Figure 3.4 Representation of the fluorescence excitation and emission spectra of the used dyes. 

a. Spectra of the intracellular calcium chelator Fluo-8AM, CellTracker Orange CMRA and membrane dye PKH26 obtained using ThermoScientific Fluorescence SpectraViewer. Note that, despite having a very similar fluorescence spectrum, alternative products (Alexa Fluor 488 and Alexa Fluor 555) were used in this graphic, since Fluo-8AM and PKH26 are not on the seller database. 
b. Excitation (green) and emission (red) spectra of CalciFluor Fluo-8AM (Santa Cruz BioTechnology). 
c. Excitation and emission spectra of PKH26 dye (Sigma-Aldrich).

3.4 Flow Cytometry Data Collection and Analysis

Flow cytometry experiments were run using either BD Accuri™ C6 flow cytometer or BD FACSCanto™ System. Samples were run at medium speed, with a limit on the event number, on the total time and on the total sample volume depending on the experiment. These limits were calculated knowing the sample concentration (usually $10^6$ cells/ml) and
the device flow rate (e.g. BD Accuri™ C6 flow cytometer has a flow rate of 35µl/min at medium speed). The data were analysed with the software package FlowJo.

Gating in the experiments was done as follows. At first, on the Forward Scatter (FSC): Side Scatter (SSC) (X:Y) plot (FSC indicates the cells size, while SSC shows their granularity), the population of viable cells was selected. For studies where B3Z and K89 were mixed, no clear distinction between the cell types was visible from this graph, as they had similar sizes. Secondly, single cells and doublets were distinguished and gated on the Forward Scatter-Height: Forward Scatter-Area (FSC-H : FSC-A): cells aligned on the 45° diagonal of the quadrant were considered as single cells, while cells outside the diagonal group were labelled as doublets. Hence, B3Z were gated based on their basal green fluorescence (on the FL1/FITC histogram, before any stimulation); the threshold was adjusted on each sample at the beginning of the recording. All experiments where both dyes where added were run using FACSCanto, using the automatic compensation between red and green channels. Populations could be readily distinguished from the FL1 : FL2 (X:Y). At last, the fluorescent B3Z population was plotted against time (Time : FL1 (X:Y)) to assess any shift in MFI (hence activation state) after stimulation. This final plot allowed to quantify average percentages of activators and normalized shifts in fluorescence; as previously mentioned, these data give an average over the bulk population of lymphocytes, lacking the time resolution at a single cell level. A schematic of the gating process is reported in Figure 3.5.
Figure 3.5 Gating process on flow cytometry data. In this experiment B3Z were stimulated by K89 presenting SL8 epitope. From left to right: gate on the viable cells (comprehensive of both B3Z and K89 populations); from the viable cells selection, single cells and doublets are distinguished in the middle figure; in the last plot, B3Z population is differentiated from K89 depending on the FL1-FITC threshold. The (red) histogram represents the entire population of viable cells, the blue and the orange histograms picture the B3Z population before and after stimulation with K89 (gated on the FL1:Time plot
3.5 Photolithography for master fabrication

While the methodology focused up to now on biology protocols, it is relevant to explain in this Chapter the fabrication process of the masters used to cast the single cell trapping devices that will be introduced later on in Chapter 5: . In order to fabricate the trapping arrays, in fact, replica molding technique was used, and each device was made casting an agarose solution onto a master carrying the negative features of the final design. Masters were prepared in the Southampton Nanofabrication Centre, using photolithography. This method allows to develop 3D microstructures from a photoresist spincoated onto a substrate that will then be exposed to UV light through a mask with the design of the features. When developed, the resin will crosslink only where it has been exposed (negative photoresist) or where it was shaded (positive photoresist). Figure 3.6 reports the steps to obtain a master; the same master can be used several times (more than 20) to fabricate new devices. Its features are made of SU8 negative photoresist, and are anchored onto a glass or silicon substrate, called wafer.

![Figure 3.6 Schematic of the photolithography process steps. From the top left: TI prime is spin-coated on the selected substrate (glass/silicon wafer); TI prime is cured on a hot plate (120°C, 2 minutes); SU8 photoresist is spin-coated on the substrate; SU8 is partially cured on a hot plate (soft bake); SU8 is exposed to UV light through a black/transparent mask with the design of the final features; SU8 is cured on a hot plate (post exposure bake); uncured SU8 is washed away using EC solvent; the obtained master is hard baked on a hot plate.](image)

50
Wafers were initially rinsed with acetone and IPA, dried under a nitrogen gun and left overnight to dehydrate at 200°C: this cleaning step ensures good adhesion of the TI prime. A thin layer of TI prime was spincoated onto the wafer; this promoter enhances the SU8-substrate adhesion, preventing features to peel off with time. Different SU8 series can be used depending on the height of the features to aim for. Both TI prime and SU8 bottles were warmed to room temperature and degassed by leaving them outside the fridge with the lid slightly loose overnight, to control their viscosity and prevent air bubbles to be trapped in the layers. After spincoating the TI prime layer at 500 rpm for 5 s/ 3000 rpm for 30 s/ 100 rpm for 5 s, the wafer was baked for 2 minutes at 120°C. The photoresist was spun at different speeds and for different times depending on the SU8 used and the target height; based on the specific recipe, the wafer was then pre-warmed 65°C and soft baked at 95°C for different times, and let to cool down before moving to the mask aligner. It was then exposed to UV light to give the specific dose, and post-exposure bake at 65°C / 95°C followed. Master features were developed in EC solvent, by submerging the wafer while shaking, and rinsed in IPA. Feature profiles were checked at the profilometer, both to confirm the height of the developed layer and the quality of the development. The master was eventually hard-baked for 2 min at 150°C, to strengthen the features. Table 3.1 includes all the details (SU8 used, spin-coating speeds, baking and developing times) of the recipes used during the project, that will be referred to later in the manuscript.
<table>
<thead>
<tr>
<th>RECIPE (SU8-No)</th>
<th>HEIGHT</th>
<th>TI PRIME</th>
<th>BAKE</th>
<th>SU8</th>
<th>SOFT BAKE</th>
<th>UV DOSE</th>
<th>POST BAKE</th>
<th>RINSE</th>
<th>HARD BAKE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (3025)</td>
<td>27 µm</td>
<td>500 rpm/ 5 s 3000 rpm/ 30 s 100 rpm/ 5 s</td>
<td>120°C /2 min</td>
<td>500 rpm/ 5 s 2100 rpm/ 30 s 500 rpm/ 5 s</td>
<td>65°C/ 5min 95°C/ 15min</td>
<td>150 mJ/cm²</td>
<td>65°C/ 5min 95°C/ 10min</td>
<td>4.5min EC 1 min IPA</td>
<td>150°C /2 min</td>
</tr>
<tr>
<td>2 (25)</td>
<td>27 µm</td>
<td>500 rpm/ 5 s 3000 rpm/ 30 s 500 rpm/ 5 s</td>
<td>120°C /2 min</td>
<td>500 rpm/ 5 s 2000 rpm/ 30 s 500 rpm/ 5 s</td>
<td>65°C/ 2.5min 95°C/ 6min</td>
<td>250 mJ/cm²</td>
<td>65°C/ 1min 95°C/ 2.5min</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3 (5)</td>
<td>5 µm</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>500 rpm/ 5 s 2500 rpm/ 30 s 500 rpm/ 5 s</td>
<td>65°C/ 2min 95°C/ 5min</td>
<td>180 mJ/cm²</td>
<td>65°C/ 1min 95°C/ 1min</td>
<td>4 min EC 1 min IPA</td>
</tr>
<tr>
<td>4 (3050)</td>
<td>50 µm</td>
<td>500 rpm/ 5 s 3000 rpm/ 30 s 500 rpm/ 5 s</td>
<td>120°C /2 min</td>
<td>500 rpm/ 5 s 3000 rpm/ 20 s 500 rpm/ 5 s</td>
<td>65°C/ 2min 95°C/ 10min</td>
<td>150 mJ/cm²</td>
<td>65°C/ 2min 95°C/ 4min</td>
<td>4 min EC 1 min IPA</td>
<td>150°C /2 min</td>
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<tr>
<td>5 (3025)</td>
<td>25 µm</td>
<td>500 rpm/ 5 s 3000 rpm/ 30 s 100 rpm/ 5 s</td>
<td>120°C /2 min</td>
<td>500 rpm/ 5 s 4000 rpm/ 20 s 500 rpm/ 5 s</td>
<td>65°C/ 2min 95°C/ 10min</td>
<td>150 mJ/cm²</td>
<td>65°C/ 2min 95°C/ 4min</td>
<td>4 min EC 1 min IPA</td>
<td>150°C /2 min</td>
</tr>
</tbody>
</table>

Table 3.1 Master fabrication protocols. A number was assigned to each recipe used, to refer to were needed in the manuscript. The SU8 series used in the specific protocol is reported below the recipe number. Height refers to the aimed thickness for the patterned layer.
Chapter 4: Biological cell model

In this chapter, the cell model used in the study will be introduced and characterised.

Paragraph 4.1 will introduce the method of characterising average response of a population of cells to a stimulus by flow cytometry.

Paragraph 4.2 will investigate the response, at the single cell level, of CD8+ T cells cultured in picowell arrays to stimulation using ionomycin, an ionophore.

Paragraph 4.3 highlights individual T cell responses to a co-stimulatory factor, antiCD3. Time signals of lymphocytes stimulated with different amounts of soluble antibodies will be assessed. Soluble and beads-conjugated antiCD3 will be compared.

Paragraph 4.4 investigates individual T cell responses to antigen presenting cells loaded with a specific epitope. Protocols to capture biological activations of lymphocytes are discussed.

Paragraph 4.5 summarizes the conclusions of the Chapter.

4.1 Flow Cytometry Characterization

Flow cytometry was used to measure the bulk response of the cell population to compare to the results of single cell responses captured using single-cell arrays. Experiments have been run to optimize the protocol for the detection, to prove the feasibility of our test and to better understand cell behaviour and activation times. It is important to remember the drawback of this method, that it doesn’t allow to see individual cell activations over time. This can limit the resolution in determining whether a cell is activated or not, since it is highly likely that at the instant at which the cell crosses the laser, the phosphorylation cascade, leading to the release of Ca^{2+} from internal stores (the measured parameter) is not synchronised for all cells. Alternative methods that allow to monitor cell signals over time are limited: fluorescence microscopy per se doesn’t allow high-throughput data processing if cells are not confined in specific and highly-packed positions, and most fluorescence plate readers cannot pick the fluorescence signal of a single cell. The ability to monitor the Ca^{2+} increase in thousands of individual cells with respect to time is a major advantage of the microwell arrays.
While it was shown that some cells would activate against APC after several minutes\textsuperscript{204}, it is also important to capture cells that activate earlier. Hence, most recordings were made to capture the first 5 minutes after cell-cell contact; flow cytometry recordings as well as trapping plates time-series (see later) showed that this was a good window to pick up the first activations. The time resolution necessary for these experiments was relatively low, since most slopes in the activation signals took more than 1 second to reach their maximum.

A full activation of T cells requires orchestration of co-stimulations around the TCR-pMHC complex. Nevertheless, it is possible to induce a partial activation of the lymphocytes that leads to an hyporesponsive state called \textit{anergy}. This occurs when the TCR engagement lacks the appropriate co-stimulations, and is biologically useful to maintain an “off” state that prevents an excessive immune response e.g. to self-antigens\textsuperscript{18}. The induction of anergy seems to involve a disproportion between the calcium/NFAT signalling not sustained by other biochemical cascades like the Ras/MAP kinase pathway. This results in a transcriptional upregulation of negative regulatory proteins that prevent a correct TCR/CD28 signalling\textsuperscript{18}. Since the goal of the project was to validate a method to monitor the early stages of T cell activation, and it relied entirely on the detection of the lymphocytes’ intracellular calcium increase upon stimulation, soluble reagents have also been used to engage the T cells, despite this leading to anergy rather that fully activated states.

A well-documented way\textsuperscript{18} to induce anergy in T cells is using ionomycin. Ionomycin is a calcium ionophore that is able to bind to extracellular ions in the media and, being lipid-soluble, can transport Ca\textsuperscript{2+} across the cell membrane into the cell. Dimethyl Sulfoxide (DMSO) is generally used as a carrier for the ionophore to increase cell membrane permeability, taking care not to exceed a final 1% concentration, limit above which it can damage the cells in culture. Ionomycin also triggers the generation of IP\textsubscript{3} in the cells, prompting the release of internal stores of calcium through the IP\textsubscript{3} channels (as explained in 2.1.1). For these reasons, the intracellular calcium increase subsequent to ionomycin stimulation is the maximum that can be observed in the T cell populations, and this compound gives the opportunity to easily validate the detection system while assessing the maximum fluorescence signal expected for each experiment.

Alternatively, antiCD3 antibodies have also been tested to stimulate the cell population. CD3 is a T cell co-receptor and it consists in 4 transmembrane protein chains (CD3\textsubscript{γ}, CD3\textsubscript{δ} and two CD3\textsubscript{ε} chains) each containing an Immunoreceptor Tyrosine-based Activation Motif (ITAM) in its intracellular tail. Phosphorylation of the ITAM enables the chains to bind to the ZAP70 enzyme, a kinase that starts the lymphocyte signalling cascade. As for ionomycin, the activation obtained using antiCD3 alone leads to anergy. Nevertheless, numerous studies employed these antibodies to profile T cell activation patterns\textsuperscript{17,59,193,205–208}. These studies
employed soluble antibodies\textsuperscript{205}, antibodies conjugated to beads\textsuperscript{17,59,193,209} or antibodies micropatterned in arrays\textsuperscript{208}. Often, the antiCD3 antibody is used in combination with antiCD28, where CD28 is another T cell co-receptor specific for B7 proteins; however, previous work in our lab\textsuperscript{210} and others\textsuperscript{208} showed that the addition of antiCD28 to antiCD3 doesn’t influence the calcium responses of T cells, and since our detection system is based on the lymphocytes’ Ca\textsuperscript{2+} oscillations, antiCD28 antibodies have not been investigated in this project.

### 4.2 Lymphocytes stimulation via ionomycin

Ionomycin is an ionophore that can be used to achieve the highest intracellular calcium increase in lymphocytes, and it has been adopted to assess the maximal fluorescence range expected across all experiments. Different stimulation conditions were tested, running a titration and comparing the results in the presence/absence of extracellular calcium.

Due to the double action of ionomycin (transferring external Ca\textsuperscript{2+} into the cytoplasm and inducing IP\textsubscript{3} production leading to an opening of the ER/RER/cell membrane calcium channels), the rise in the ion concentration is instant, and the MFI peak occurs within seconds. It is also important to notice that most of the cells will present a fluorescent shift regardless their activation state, since their cytoplasmic calcium level will increase for the ion intake from the external buffer, so the percentage of activators cannot be directly compared to the one observed with biological stimulation.

In all experiments, Fluo8-AM stained B3Z were resuspended to a concentration of 10\textsuperscript{6} cells/ml; a small amount of unstained cells was routinely used as a negative control for the staining, and resuspended at the same concentration. Each sample (350 µl) was run as depicted in Figure 4.1: an initial 1.5 min recording was used to assess the basal fluorescent level; following, the recording was paused, the Eppendorf was removed from the machine and ionomycin solution was added and mixed using a pipette; the sample was then promptly put back on the holder and the recording was restarted for other 3 minutes.
Figure 4.1 Typical steps for ionomycin stimulation experiments using flow cytometry. From left to right: the sample is loaded and run for 1.5 minutes; the recording is paused and the stimulant is added and mixed to the sample using a pipette; the sample is loaded back and recorded for other 3 minutes. The fluorescent shift will be immediate; hence the sample must be handled quickly after ionomycin addition.

4.2.1 Ionomycin Titration

In this study, the B3Z population was stimulated with different concentrations of ionomycin. The experiment has been repeated on different days, with different cell batches and using different flow cytometers. Samples were run at medium speed (35 µl/min on the BD Accuri™ C6 and 60 µl/min on the BD FACSCanto™) for a total of 4.5 minutes (1.5 minutes before and 3 minutes after stimulation). This would give:

<table>
<thead>
<tr>
<th>Acquisition (min)</th>
<th>Flow Rate (µl/min)</th>
<th>Volume (µl)</th>
<th>Concentration (cells/ml)</th>
<th>Events (cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>35</td>
<td>156</td>
<td>10^6</td>
<td>1.6*10^5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>270</td>
<td></td>
<td>2.7*10^5</td>
</tr>
</tbody>
</table>

Hence, limits were set as follows: 4.5 on the total time, 200 (/300) µl on the total sample volume and 2 (/3) *10^5 on the total event number. Each sample consisted of 350 µl. After 1.5 min recording, when the stimulant was added, it was assumed that 300 (/250) µl were left in the Eppendorf, and concentrations were calculated on that volume. The stock of ionomycin calcium salt (1 mM/ MW 747,07/ 1 ml DMSO; I3909, Sigma-Aldrich) was diluted in PBS to make the desired concentrations. 3 (/2.5) µl of ionomycin solution were added to each 300 (/250) µl sample (1:100).

As reported in literature^{125}, low concentrations of ionomycin (100 nM = 74.7 ng/ml) primarily act discharging the ER calcium stores, while high ionophore concentrations (10 µM = 7470 ng/ml) dissipate the ion gradient across the plasma membrane. As such, the experiments were run spanning across a similar range (from 50 ng/ml to roughly 10 µg/ml).
This is consistent with the concentrations tested from other groups\textsuperscript{175,211–213}. After the usual gating process detailed in 3.4 (viable cells/ single cells/ above FL1 threshold), the data analysis was done as follows. On the Time:FL1 plot, B3Z were gated before and after stimulant addition, and for each gate the Mean Fluorescence Intensity value was calculated (MFI\textsubscript{0} is the basal fluorescence level, while MFI indicates the mean value after the stimulation). Hence, the normalized percentage of fluorescence increase was determined as \((\text{MFI}-\text{MFI}_{0})/\text{MFI}_{0} \times 100\); this was considered a better indicator compared to absolute values, to dissociate from the basal fluorescence decrease observed between samples over time. As shown in Figure 4.2, results are consistent with expectations. Higher concentration of ionomycin induce a greater normalized increase in fluorescence, that corresponds to a higher level of intracellular calcium. As suggested by the fit (red line), the increase seems to reach a plateau at higher ionomycin doses, as likely the intracellular calcium level tends to balance the extracellular concentration. All experiments were run in RPMI, having a nominal calcium concentration of 0.42mM (as reported in Sigma-Aldrich website, RPMI-1640 Media Formulation).

![Figure 4.2 Ionomycin titration results](image)

Figure 4.2 Ionomycin titration results. Each triangle indicates a tested sample; samples are merged from different experiments, comprising both technical and biological replicas. The fluorescent increase after ionomycin stimulation was normalized on the basal level of the sample before ionophore addition. The dose-response curve was fitted using the symmetrical sigmoidal function from GraphPad Prism (\(R^2 = 0.90\)), assuming that at high concentrations of ionomycin, the intracellular calcium will tend to balance the ionic concentration in the buffer.

Data were also processed to analyse the percentage of B3Z activators in response to the ionomycin titration. In the Time:FL1 graph, a threshold was set to the highest level of MFI\textsubscript{0};
all events after the stimulation were gated and counted, and all events above threshold from the same gate were also quantified, and considered as activators. Percentages of activators for the tested concentrations were found to be:

<table>
<thead>
<tr>
<th>Ionomycin (ng/ml)</th>
<th>Activators (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>95.57</td>
</tr>
<tr>
<td>500</td>
<td>86.30</td>
</tr>
<tr>
<td>200</td>
<td>75.10</td>
</tr>
<tr>
<td>100</td>
<td>73.86</td>
</tr>
<tr>
<td>10</td>
<td>26.69</td>
</tr>
</tbody>
</table>

While almost all cells activate at higher concentrations of ionomycin, in the lower range fewer cells respond to the stimulus; a hypothesis to explain this behaviour could be that in this range the active status is determined by the IP₃ cascade initiation mainly, hence by the sensitivity of the single cell rather than to a simple dissipation of a calcium gradient across the cell membrane.

An experiment was run to assess this hypothesis. Each sample was stimulated twice using ionomycin; the first time using a different concentration depending on the sample (titration), while the second time using the highest concentration of the ionophore (1000 ng/ml). Recordings comprised initial 1.5 minutes to assess the basal fluorescence and 2 minutes following each stimulation. All samples were resuspended in RPMI. Limits on the fluidics were calculated as follow:

<table>
<thead>
<tr>
<th>Acquisition (min)</th>
<th>Flow Rate (µl/min)</th>
<th>Volume (µl)</th>
<th>Concentration (cells/ml)</th>
<th>Events (cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>35</td>
<td>193</td>
<td>10⁶</td>
<td>1.9*10⁵</td>
</tr>
</tbody>
</table>

As shown in Figure 4.3, for high concentrations of ionomycin in the first stimulation, the second stimulus does not make a great effect; this is due to the fact that the calcium gradient across the cell was already dissipated. For samples in which the ionophore concentration was initially low, the second trigger had more resonance. In this range, the depletion of the intracellular calcium stores has a higher impact.
4.2.2 External Calcium Influence

To further investigate the influence in the choice of the buffer in the experimental protocol, due to its extracellular calcium concentration, an experiment has been run in different conditions: with cells resuspended in growth media, in plain RPMI and in calcium-free PBS.

RPMI has a calcium concentration of 0.42 mM (Ca(NO₃)₂·4 H₂O content of 0.1 g/L, molar mass 236.15); the only supplement added in the growth medium that contained Ca²⁺ was the FBS. Despite the variability between batches and products, calcium concentration in FBS was found to be 3.5 – 4 mM according to literature²¹⁴. Adding 50 ml FBS (7.0137 – 8.0156 mg Ca²⁺) to a total 515.5 ml solution 0.407 mM (8.4164 mg Ca²⁺), it was therefore assumed that the culture medium (referred to as *full*), had a calcium molarity in the range 0.681 – 0.725 mM. As a calcium-free control, cells were alternatively resuspended in PBS (DPBS, no calcium, no magnesium; ThermoFisher Scientific, 14190359).
For each sample, the same stimulation pattern was repeated: the basal fluorescence level was recorded and then ionomycin was added; after 5 minutes, the MFI was recorded pre/post a second addition of stimulant; a third ionomycin addition was repeated after 10 minutes. Following, the fluorescent level of the population was checked at 5 and 10 minutes from the last stimulus. The ionomycin stock used was 100 µM and it was added 1:100 v/v each time, hence the ionophore concentration in the sample ranged from 1 – 3 µM (747 – 2241 ng/ml). The stimulation range was selected to capture the range in which the response should be mostly linked to the calcium concentration of the media.

Results are shown in Figure 4.4. While the populations responded similarly to the first ionomycin stimulation, the difference in the presence or absence of calcium in the extracellular environment changed the following behaviour: while the MFI was sustained when the buffer contained ions, the intracellular calcium concentration dropped to the initial level in DPBS, despite the subsequent stimuli. This suggests that the first rise was actually related to the activation of the IP₃ cascade, and the subsequent calcium release; in the case of sustained response, the MFI slightly dropped after the second ionomycin addition, suggesting an opening of the calcium channels in the mitochondria, that likely started to sequester the intracellular excess (indeed both the second and third stimuli didn’t result in any sharp fluorescent increase). As shown in Figure 4.5, all samples showed a similar timescale to restore the basal intracellular calcium level (10-15 minutes), with longer estimated times when the ionophore could still bring ions across the cells membrane. The unresponsiveness of the DPBS population shows the refractory period for lymphocytes to activate within a short time frame.

![Graph](image)

Figure 4.4 Multiple ionomycin stimulations on B3Z cell samples resuspended in common buffers containing different calcium levels. Growth media (full), plain RPMI and calcium-free...
DPBS were tested. Vertical lines correspond to the times at which ionomycin was added (each time 1:100 v/v from a 100 µM stock); the horizontal red line marks the initial MFI. In the absence of calcium ions in the buffer, B3Z didn’t respond to the stimuli, and restored the basal MFI, showing a refractory period longer than 10 minutes. Lymphocytes resuspended in media sustained a higher fluorescent level, that started to drop after the second stimulation (likely due to the sequester of intracellular free calcium to restore the basal level).

Figure 4.5 Basal intracellular calcium level recovery. Data adapted from Figure 4.4. MFI level in all B3Z populations after the initial drop was merged to simulate the decrease in absence of further ionomycin stimuli. Within 10/15 minutes (when the ionophore could still bring ions across the cytoplasmic membrane), the basal fluorescent level was restored.

4.2.3 Correlation between subclone sensitivity and ionomycin response

Cahalan’s group (University of California, Irvine) studies the immune response at a single cell level using patch-clamp techniques, and has been characterizing the calcium activity of B3Z lymphocytes in the past. Specifically, the group assessed the correlation between the calcium oscillations upon stimulation using antiCD3 and thapsigargin (TG) and the lacZ production on single B3Z. TG generates a sustained influx of Ca²⁺ by inhibiting the sarco/endoplasmic reticulum calcium transport ATPase (SERCA) and preventing the filling of the intracellular calcium stores. LacZ activity was evaluated after 4 hours of stimulation staining the cells with Fluorescein di(β-D-galactopyranoside) (FDG). Their paper suggests that NFAT-regulated lacZ activity is all-or-none, and that the gene expression is correlated to the intensity of the intracellular calcium concentration signal. In detail the authors state that, while the amplitude and frequency of responses were heterogeneous in response to
immobilized antiCD3, when a higher average in the calcium level was reached, cells were more likely to be lacZ+. Also with TG stimulations, the changes in intracellular calcium levels affected the percentages of activators (with a 15-fold increase in the free Ca\(^{2+}\) level from the basal 70 nM, 50% cells activated). Cells required a sustained signal for >25 min before lacZ expression became detectable, and authors suggest that the intracellular calcium concentration required for activation lies in the range of 200 nM – 1.6 µM. Considering that ionomycin acts similarly to TG, an experiment was run to determine whether there was a correlation between the population sensitivity and their intracellular calcium range (e.g. a variability in the basal calcium level that would favour a faster reaching of the threshold of activation, or a different initial calcium intake). Five sensitive and five non-sensitive B3Z populations were selected from a subcloning assay run by Dr Emma Reeves; for each sample basal fluorescence was recorded before and after stimulation with ionomycin (1:50 v/v from a 100 µM stock). On the same day, responsive populations were tested to confirm their sensitivity to SL8 epitope. Results are shown in Figure 4.6. No differences could be highlighted between the intracellular calcium levels of the populations before or after the stimulation with respect to sensitivity, neither normalizing the free ions increase (MFI/MFI\(_0\)). These findings are in accordance with the belief of the authors that the final gene expression could be related to a longer time frame of the calcium response (and to the characteristic secondary calcium increase). One long-term aim of my project was to determine whether the fate of early-activating T cells (in terms of their functional phenotype which develops over hours-days after activation) is different from late or slow-activating T cells; and whether in the context of competition between T cells with different specificities, these early events program immunodominance.
Figure 4.6 Comparison between the ionomycin responses and sensitivity of subclones populations. B3Z subclones were screened as for protocol reported in 3.2 against K89 in presence of 10 nM SL8 and results are presented in the top figure. Red bars represent sensitive populations; 5 sensitive and 5 not responsive subclones were selected, and are highlighted by the arrows. The selected populations were calcium-stained and screened using flow cytometry to assess their basal fluorescence and their MFI after stimulation with ionomycin added (1:50 v/v from a 100 µM stock); results are shown in the first two images on the bottom. MFI ratio between pre and post stimulation signal is reported. On the same day, the 5 responsive (red) populations were tested for sensitivity according to the protocol reported in 3.2.1, and results are reported in the right image on the bottom. The sensitivity assay data were kindly provided by Dr Emma Reeves.
4.3 Lymphocytes stimulation via antiCD3

CD3 is a co-receptor specific of T cells that consists of 4 transmembrane proteins, each having an intracellular tail with an ITAM; when ITAMs are phosphorylated, they bind to the ZAP70 enzyme, initiating the lymphocyte signalling cascade. AntiCD3 antibodies have often been used to study lymphocytes in their early stages of activation\textsuperscript{17,59,193,205–208}. These studies employed soluble antibodies\textsuperscript{205}, antibodies conjugated to beads\textsuperscript{17,59,193,209} or antibodies micropatterned in arrays\textsuperscript{208}.

The stimulation protocol was similar to the one used with ionomycin: calcium-stained B3Z were resuspended at $10^6$ cells/ml; each sample was run for one minute to assess the basal fluorescent level; following, the recording was paused, the Eppendorf was removed from the machine and the antibody solution was added and mixed using a pipette; the sample was then promptly put back on the holder and the recording was restarted for other 4 minutes; it was then paused a second time to add ionomycin, hence the sample was put back to register for (at least) a final minute. Ionomycin was usually added at the end of each recording to assess the fluorescence range that could be reached by the cells population under the specific conditions of the experiment. As usual, data processing was done using FlowJo, and a typical time response of the lymphocytes is reported in Figure 4.7. In this case, to help the visual inspection of the trace, the recorded trace was also plotted in a Time:MFI (X:Y) graph.

Differently from ionomycin, where the cell response was immediate, antiCD3 soluble antibodies elicited a calcium increase with a usual delay of roughly 30 seconds. This pattern was consistent in all the experiments, and it is likely linked to the different way antiCD3 stimulates the cells: in this case a receptor-ligand interaction needs to occur at the cell surface to initiate the activation cascade. A similar pattern was reported in literature\textsuperscript{216} despite the different timescale (the peak was reached after 300s) that might be related to the different cell line and antibodies. Even at the peak time, it is possible to see a broad range of activations. While the external calcium uptake following ionomycin stimulation groups the responses into a relatively tight band, the simple stimulation of the CD3 complex (already more representative of a biological event) leads to a greater variation. It is noticeable that only a percentage of the B3Z is activated, and the response to the stimulus appears to be of the on-off type. Nevertheless, the exact percentage of activators can’t be directly assessed at this stage as the single cell signals can’t be followed over time using flow cytometry, and while giving an idea on the average time response, this technique fails in giving an insight on outliers that might have peaked slightly earlier or delayed compared to the average
population.

![Figure 4.7 Typical time response of a B3Z population stimulated initially with antiCD3 and following with ionomycin. On the left side, the scatter plot of the population over time; on the right side, the Mean Fluorescence Intensity of the same population is represented over time. The vertical red-dotted lines mark the time of addition of 50 ng/ml soluble antiCD3; the vertical black-dotted lines highlight the instant of 1 µM (747 ng/ml) ionomycin addition. These graphs are from a single population, while being representative of the usual results obtained in several experiments.

4.3.1 Soluble antiCD3 Titration

In this study, the B3Z population has been stimulated with different concentrations of soluble antiCD3 antibodies (anti CD3 antibody, ThermoFisher, eBio500A2). The experiment has been repeated with technical and biological replicates. Samples were resuspended at a 10^6 cells/ml concentration and run at medium speed (35 µl/min on the BD Accuri™ C6) for a total of 5 minutes comprehensive of a final stimulation using 1µM ionomycin (1 minute before, 3 minutes after antiCD3 stimulation, 1 minute after ionomycin stimulation).

After 1 min recording, when the stimulant was added, it was assumed that 165 µl of the initial 200 were left in the Eppendorf, and concentrations were calculated on that volume; ionomycin concentration was calculated on the 60 µl estimated to be in the tube after 4 minutes of recording. The stock of antiCD3 antibody (500 µg/ml) was added to the sample to make the desired concentration (starting from 1:5 v/v). The experiments were run spanning between a final 100 µg/ml and 5 µg/ml antiCD3 in the sample. The used concentrations were higher than the ones adopted in literature^{205,217,218} and suggested by the product protocol (≤ 5 µg/ml), as for lower concentrations no signal was observed in previous
experiments (data not shown). This is in accordance with the concentrations used in previous work in our lab, where B3Z were stimulated with 20 µg/ml antiCD3.

After the usual gating process detailed in 3.4 (viable cells/ single cells/ above FL1 threshold), the data analysis was done as follows. On the Time : FL1 plot, B3Z were gated before and after stimulant additions (hence before, after antiCD3 and after ionomycin). Since in the case of ionomycin, the response of the cell population was a higher fluorescent signal that was mostly stable in the few minutes of assessment, the value that was considered both for the basal signal and the ionomycin response were the Mean Fluorescence Intensities (MFIo as basal fluorescence, while MFIono indicates the mean value after the stimulation). Differently, as depicted in Figure 4.7, the response to antiCD3 antibodies gave a signal that was rapidly changing over time; in this case, in order to quantify the response to the stimulant, the Peak Fluorescence Intensity value was calculated from the averaged time response (MFIpeak).

Hence, the normalized percentage of fluorescence increase was determined in two possible ways. The first representation mimics the one previously used to depict the ionomycin responses: (MFIpeak-MFIo)/MFIo*100. Results are reported in Figure 4.8. The second representation wants to compare the fluorescence shifts obtained from antiCD3 VS the one obtained from ionomycin (expected to be the maximum that can be obtained). In this case the responses are quantified as (MFIpeak-MFIo)/(MFIono-MFIo)*100, and reported in Figure 4.9.

Results are consistent with expectations, a higher concentration of antibody induces a greater normalized increase in fluorescence, that corresponds to a higher level of intracellular calcium. The red line (in both figures) depicts the average between the replicates.
Figure 4.8 Soluble antiCD3 titration. Each triangle indicates a tested sample; samples are merged from different experiments, comprising both technical and biological replicas. The peak of the fluorescent increase after antiCD3 stimulation was normalized on the mean basal level of fluorescence of the sample. The red line interpolates the mean values between the replicas.

Considering that the antiCD3 antibody ultimately elicits the release of calcium from the internal stores and uptake of extracellular calcium, but in the absence of ionomycin the ions are not actively conducted across the cell membrane, it is expected that the cell population response to antiCD3 will be lower than the one against ionomycin. The difference was quantified for the various antibody concentrations, and shown in Figure 4.9. From the graph, it appears that the B3Z response to \( \geq 20 \mu g/ml \) antiCD3 has a plateau at roughly 60% of the maximum fluorescent shift obtained with ionomycin. For these concentrations, the fluorescent shift is actually visually distinguishable considering the entire population on the FL1/Time plot (hence it is not an artefact due to the analysis, that considers the MFI\text{peak} following antibody stimulation and could be biased by outliers). These two considerations corroborate the hypothesis that the response observed against 20 \( \mu g/ml \) antibody actually represents the physiological peak of intracellular calcium, and that the remaining 40% of the calcium response read after ionomycin stimulation might actually be linked to the active intake of ions due to the ionophore.
4.3.2 Comparison between soluble and bead-conjugated antiCD3

In literature, many research groups showed how they used antiCD3 antibodies conjugated to microbeads as an alternative to soluble antibodies, especially testing microfluidic chips. Although the microbeads diameter is not usually specified in the papers, pictures show that the selected microparticles size is slightly smaller than the lymphocytes in study. It also has been suggested that coated beads evoke a greater cells expansion compared to soluble antibodies. This experiment aimed to assess any improvement/difference when using cross-linked antibodies in the B3Z stimulation, and was initially planned to investigate the possibility of using antibodies-coated beads within the microwell array (see later).

Streptavidin-functionalized superparamagnetic fluorescent polystyrene microbeads were purchased from MicroParticles GmbH (PS-MAG-FluoRed-SA-S2467). Their average diameter (10.31µm) was slightly smaller than the average B3Z size (see 5.1.1), in accordance with literature experiments. Biotinilated antiCD3 antibodies were purchased from BioLegend (145-2C11, 0.5mg/ml).

Figure 4.9 Soluble antiCD3 titration, comparison with the maximum fluorescent shift, detected with ionomycin. Each triangle indicates a tested sample; samples are merged from different experiments, comprising both technical and biological replicas. The maximum fluorescence increase after antiCD3 stimulation was normalized on the mean fluorescence shift after ionomycin addition to the sample. The red line interpolates the mean values between the replicas.
Calcium-stained B3Z were resuspended in media to a concentration of 0.25*10^6 cells/ml; each sample consisted of 200 µl of cell suspension, and was run at medium flow for a total of 4 minutes (1 minute to record the basal fluorescence level, 2 minutes following stimulant addition and a final minute after ionomycin addition). Beads were diluted 1:5 in PBS from a starting concentration of 4.7*10^6 beads/ml (measured with flow cytometry), and for each cells sample 50 µl of the diluted beads suspension were taken, to have a final 1:1 cell:bead ratio. The microparticles were pelleted and resuspended in 15 µl of a solution of PBS containing 0.1%v/v Tween; they were washed in the same buffer for 3 times, before addition of 30 µl of a 100 µg/ml stock of biotinylated antiCD3 antibodies. Hence, beads were incubated with the antibody for 1 hour at room temperature on a shaker. Before addition to the cells sample, beads were washed once, and the supernatant from the wash was collected separately, to assess for any unbound antibody floating in the suspension. Ionomycin was added to the samples at the end of the run, to make a final concentration of 10 µg/ml. Controls were: B3Z + soluble antiCD3 (final 50 µg/ml), B3Z + biotinylated antiCD3 only (final 50 µg/ml), B3Z + beads only.

![Graph](image)

Figure 4.10 Comparison between soluble and beads-conjugated antiCD3 antibodies. The same dataset is pictured in the two graphs, obtained using the two analysis described in 4.3.1: on the left, the MFI peak after antibody stimulation is expressed as a percentage of the mean activation obtained using ionomycin; on the right the MFI peak is normalized on the basal fluorescence of the sample before stimulation. aCD3 represents a stimulation using 50 µg/ml of the soluble antiCD3 used in 4.3.1 (reference); aCD3-biotin relates to a stimulation with 50 µg/ml of the biotinilated antibody used in this experiment (control); beads represents the signal recorded after addition of streptavidin-functionalized microparticles to a 1:1 cell:bead ratio (control); beads + CD3-biotin indicates the actual signal after exposure to beads co-incubated with biotinylated antiCD3; supernatant represents the response to addition of the supernatant recovered from the beads wash after the co-incubation, as a control for unbound antibody.
In Figure 4.9, results from the experiment are reported. The comparison between the signal obtained from the soluble antibody used in 4.3.1 (aCD3) and the biotinylated one (aCD3-biotin) show that the former gave remarkably stronger B3Z activations per se. Controls (beads and supernatant) show that, while the antibody was likely bound to the beads (supernatant signal lower than aCD3-biotin), cells failed to bind to the microparticles, and the stimulation protocol should be revised. In retrospect, a vigorous vortexing of the sample after addition of the beads was not sufficient to efficiently push the cells together against the beads.

4.4 Lymphocytes stimulation via Antigen Presenting Cells

After exploring stimulations of lymphocytes with co-stimulators, activation against Antigen Presenting Cells (APCs) has been investigated. The preceding studies defined a range of time, fluorescence and averaged pattern of responses that provides the platform for designing more physiologically relevant means of stimulating T cells. I therefore progressed to measuring T cell stimulation at the single cell level using live antigen presenting cells expressing cognate peptide:MHC I complexes.

In order to implement an APC stimulation study on single lymphocytes, it was necessary to optimize the stimulation protocol using flow cytometry on the bulk population, both to have a reference for the average behaviour of the cell lines, and to have a proof of concept that the cell model in use was suitable for the platform validation. As reported in 3.1, B3Z is a T cell hybridoma known to respond, among other epitopes, to SIINFEKL (SL8) and SIINFEHL (SHL8) bound to the H-2K\(^b\) complex presented on the cell surface of some APCs, like K89. In the same paragraph it was reported how the cell lines have already been tested using colorimetric sensitivity assays based on the lacZ gene expression of activated B3Z. Nevertheless, being interested in the early activation events, it was necessary to confirm that the cell model was also appropriate when investigating the calcium fluctuations in the first minutes after stimulation.

Deriving a suitable protocol to perform this set of experiments required substantial optimisation.
4.4.1 Capture of the T cells biological activation via flow cytometry

While only the intracellular calcium staining of B3Z was necessary for the experiment purpose, as it was possible to exclude the APC cells from the analysis by gating the T cell population in the FL1 histogram, the first few experiments using flow cytometry (and all of the experiments using the microfluidic rig) were run staining both cell lines. This helped localizing the K89 population initially, and quantifying the B3Z-K89 doublets in the activation assays while optimizing the protocol. Cells were stained according to the protocols reported in 3.3.

In order to prove that the K89 membrane dye didn’t compete for sites reserved for the epitopes, and that it didn’t interfere with the analysis by biasing the peptide concentration presented on the cells surface, a colorimetric sensitivity assay was run comparing the B3Z responses to the same population of K89/ same peptide loading in the presence and absence of membrane dye. Results are reported in Figure 4.11, and show that the staining has no influence. These data are also important as they show that both peptides work equally well in eliciting the T cells responses, and could be used interchangeably in the experiments.

Figure 4.11 Comparison of B3Z responses to peptide-loaded K89 in the presence or absence of PKH26 staining. K89 have been pulsed with the two peptides that were used in this project (SIINFKL, SL8, and SIINFEHL, SHL8) at different concentrations, and co-incubated with B3Z as for protocol reported in 3.2.1. On the x-axis, 0 indicates the control (K89 without peptide). LacZ expression of the activated B3Z was assessed with a colorimetric assay.
After staining the cells, K89 were incubated in media containing 10 µM peptide for 1 hour to let the peptide bind to the H-2K\textsuperscript{b} complexes on the cell surface. Following, unbound peptide was washed off from the solution with a centrifuge step (290 g, 5 min). A control population of K89 not loaded with peptide was always prepared in parallel.

The first set of experiments to capture the B3Z activation against SL8/SHL8-pulsed K89 were run according to a protocol previously used in our laboratory\textsuperscript{210}. B3Z were resuspended at 10\textsuperscript{6} cells/ml and run at medium flow for one minute to assess the basal fluorescence before adding the APCs. K89 were counted to have 10:1 APC:T cell when mixing to the T cells sample. In order to increase the likelihood of TCR:pMHC engagement by forcing the cells into contact, the mixture was also centrifuged at 900 rpm for 3 minutes at 4°C. Following, the sample was mixed with a pipette and put back on the rack to run for a further ≥ 2.5 minutes to capture the activations. A final stimulation with ionomycin was used to prove the fluorescence range of detection. In Figure 4.12, the typical steps of the data acquisition of these experiments are summarized.

![Diagram of cell stimulation steps](image)

**Figure 4.12** Typical steps for APC stimulation experiments using flow cytometry. From left to right: the sample is loaded and run for 1 minutes to detect the basal fluorescence; the recording is paused and the APCs are added at a 10:1 cells ratio; the Eppendorf is centrifuged to create the cell-cell contact; the sample is resuspended, loaded back and recorded for other 3 minutes. An optional final stimulation with ionomycin helps assessing the fluorescent range that can be detected in the specific sample.

Former studies\textsuperscript{210} reported that the ratio between the two cell lines and the additional low temperature spin played a major role in enhancing the probabilities of T-cell/APC contacts. The low temperature was used to help cells maintaining their spherical shape, likely to guarantee a greater exposure of the binding sites. Since no activations could be observed when using the same protocol, some alternative methods sharing the same rational were tested. Firstly, the B3Z/peptide-loaded K89 sample was left to settle for 5 minutes on ice;
secondly, the sample was left on a rotatory platform in a cold room for 5 minutes; at last, the sample was tested combining the centrifuge step and a further 3 minutes for settling. In all cases, no activations were observed. Note that the volumes in which cells were resuspended were not specified in the previous protocol; as such, in order to guarantee a better viability of the cells throughout the experiment, K89 were maintained at a $10^6$ cells/ml concentration in media, and aliquots from the stock were directly added to the B3Z samples. Hence, in order to maintain the 10:1 ratio, K89 were added in 10 times the media volume of the initial B3Z sample. This proved to be the first issue, especially in the sedimentation incubation. Cells sedimentation velocity was experimentally quantified in literature$^{220}$. The group tested a cell line having a very similar size to the ones in use (14.5µm diameter), and showed that their settling velocity was 3.5cm/h (0.58mm/min). Assuming similar test conditions for the present cells co-incubation and considering a 1.5ml Eppendorf, a cell on the top layer of the 1.5ml suspension would virtually take (22mm * 0.58mm/min =) 13 minutes to reach the bottom; if the initial volume was 250µl, it would take (8mm * 0.58mm/min=) 5 minutes only.

Note that the cells ratio and the sample volume shouldn’t play a big role in enhancing the likelihood of cell-cell contact when using a centrifuge. This can be proved approximating the time that cells take to reach the bottom of the falcon tube/microcentrifuge tube during the spinning. In order to do so, it is necessary to balance the forces involved in the centrifugation, depicted in Figure 4.13: centrifugal force ($F_c$), frictional force ($F_f$) and Buoyant force ($F_b$).

![Figure 4.13 Schematic of the forces involved in centrifugation: centrifugal force ($F_c$), frictional force ($F_f$, force generated by the cells while migrating through the media) and Buoyant force ($F_b$, force exerted by the cells to displace the media where they sediment). The circle represents a cell in the furthest position from the bottom of the collection tube; $\omega$ represents the centrifuge angular velocity.](image-url)
The centrifugal force applied to the sample is dependent on the centrifuge rotor and selected speed, and can be expressed as \( F_c = \omega^2 rm_p \) (\( \omega = \) angular velocity, \( r = \) rotor radius, \( m_p = \) cell mass). The Buoyant force can be expressed as \( F_b = -\omega^2 rm_s \) (\( m_s = \) media mass displaced by the cell). The frictional force can be expressed as \( F_f = -fv \) (\( v = \) velocity, \( f = \) frictional coefficient). As such, the forces equilibrium can be expressed as:

\[
\omega^2 rm_p - \omega^2 rm_s - fv = 0
\]

4.1

Considering the following substitutions

- \( \omega = \frac{60}{\pi} RPM \)
- \( m_p = \rho_p V_p = \rho_p \frac{4}{3} \pi r_p^3 \), where \( \rho_p, V_p \) and \( r_p \) are the cell density, volume and radius
- \( m_s = \rho_s V_p = \rho_s \frac{4}{3} \pi r_s^3 \), where \( \rho_s \) is the media density
- \( f = 6\pi \eta r_p \), where \( \eta \) is the media viscosity
- \( v = \frac{dr}{dt} \),

Equation 3.1 can be rephrased as \( \frac{dr}{dt} = Cr \), having \( C = \frac{2r_p^2(\rho_p - \rho_s)}{\eta} \times \left( \frac{60}{\pi} RPM \right)^2 \). This differential can be solved as \( r(t) = r_0 \times e^{Ct} \), and the time at which a cell initially positioned in \( r_0 \) will reach the bottom of the tube (position \( r_{max} \)) can be calculated as \( t = \frac{1}{C} \ln\left( \frac{r_{max}}{r_0} \right) \).

For a rough estimate, the values to substitute in the equation can be found in literature:

<table>
<thead>
<tr>
<th>cell diameter ((2r_p))</th>
<th>13 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell density ((\rho_p))</td>
<td>1080 kg/m³ [221]</td>
</tr>
<tr>
<td>media density ((\rho_s))</td>
<td>990 kg/m³ [121]</td>
</tr>
<tr>
<td>media viscosity ((\eta))</td>
<td>(0.78 \times 10^{-3} ) Pa*s [121]</td>
</tr>
<tr>
<td>speed ((\text{RPM}))</td>
<td>900 rpm</td>
</tr>
<tr>
<td>centrifuge rotor ((r_{max}))</td>
<td>18 cm (Eppendorf A-4-81) 8.3 cm (Eppendorf F-45-12-11)</td>
</tr>
<tr>
<td>tube height ((r_{max} - r_0))</td>
<td>11.9 cm (15ml Falcon) 4 cm (1.5ml Eppendorf)</td>
</tr>
</tbody>
</table>

For both centrifuges used in this study, once the speed is at regime, cells will reach the bottom of the tube in far less than 1s (\( t_{max} = 13.5 \) ms for the big centrifuge and \( t_{max} = 8.2 \) ms for the smaller one). Certainly, these results are a very broad approximation, not accounting for the cells interactions. Anyhow, it is very unlikely that the lack of activations was due to the low chances of interactions between cells, even at low centrifugation speed.
A further experiment confirmed that the lack of responses wouldn’t change with increasing the incubation times during sedimentation. The rational was to reproduce the co-incubation conditions employed in the sensitivity assay (see 3.2.1), that proved that the B3Z would eventually activate, having produced lacZ gene after 6 hours of incubation. For each well of a flat-bottomed 96-well plate, $10^5$ B3Z and $10^5$ K89 were resuspended in a total of 200 µl of growth media, in the presence or absence of 10 µM SHL8. Seven samples containing peptide and seven blanks were prepared in different wells of the plate. Each 7-samples set was loaded using a multipipetter, to have the same time-zero of co-incubation. Samples were then run one by one on the flow cytometer, one every 5 minutes in order to have 0-5-10-15-20-25-30 minute time points at which the activation state of the B3Z population was assessed. The experiment was repeated in two different days, the first time running the peptide-loaded samples first and the second time starting from the blank samples; this would prevent any bias in the readings due to degradation of the fluorescent dye over time. Considering the average radius of a well in a flat bottomed 96-well plate (3.19 mm), and having a volume of 200 µl (200 mm$^3$), the top layer of the sample would be at a height of approximatively 6.3 mm from the bottom. According to the rough estimation earlier introduced, cells would take an average of 3.6 minutes to reach the bottom of the well, and so a fluorescent shift would be expected starting from the first sample. Results are shown in Figure 4.14.
Figure 4.14 B3Z biological stimulations time points using flow cytometry. The top four graphs represent the overlaid FITC histograms of each sample; the first two belong to the experiment in which the blank was run before the set of samples loaded with SHL8; the third and fourth graph include the data from the second trial, in which the peptide loaded samples were analysed before the control population. The four bottom graphs are the staged version of the data on top, to better visualize the histogram shapes of each sample for comparison.

While a fluorescent decay is observable over all the populations and it is likely due to degradation of the loaded fluorescent dye, it is also noticeable that a second fluorescent peak is not marked in any of the samples; nevertheless, samples loaded with peptide (in both experiments) had a wider fluorescent range. This can be due to cells activating in an unsynchronized manner; in fact, sedimentation doesn’t allow to control the time of cell-cell contact over the entire population. If the calcium response is not sustained to a constant level over time, the asynchronous fluctuations over the entire population will add up to break the distribution into a wider curve. One of the main requirements (and advantages) of the microfluidic platform is indeed that it allows a synchronous stimulus of the lymphocytes to
better define their responses and spot outliers. Another trivial deduction from these results is the time limit imposed to the data acquisition window, due to dye degradation.

4.4.2 Antigen presenting cells selection and characterization

In order to confirm that K89 was the best candidate for B3Z activation, two sets of experiments have been run. Firstly, different antigen presenting cell lines were tested with a sensitivity assay and by staining both the H-2K\(^b\) complexes and the H-2K\(^b\) complexes conjugated to SL8 after 1h incubation with peptide; secondly, SL8 levels exposed on the K89 surface were assessed over time, to evaluate the optimal protocol to pulse the antigen presenting cells prior to mixing with B3Z.

Antigen presenting cells were selected among the ones presenting the correct docking site for the selected epitope. Specifically, the following cell lines were investigated: EL4 (mouse thymoma), FSDC (murine dendritic cells) and B16 (murine melanoma cell line). Two sensitivity assays were prepared as for protocol in 3.2.1 using an higher (5 µM) and a lower (100 nM) starting concentrations of SL8. In both cases, K89 resulted to be the best candidate for B3Z stimulation due to the higher responses, consistent across the peptide concentrations range (see Figure 4.15).

![Figure 4.15 Sensitivity assays comparing different APC cell lines. Starting SL8 concentrations were 5 µM (left) and 100 nM (right). Investigated cell lines were K89, EL4, B16 and FSDC; all stimulations were against B3Z cell line. FSDC data are missing for the lower concentration assay, due to the slow growth rate that resulted in fewer cells for the assays.](image)

The relative amounts of H-2K\(^b\) and of H-2K\(^b\)/SINFEKL (after populations were pulsed with 10 µM SL8 for one hour) were compared among the cell lines by fluorescent antibody staining and flow cytometry analysis.
For each cell line four samples, each containing $2.5 \times 10^5$ cells, were prepared: unstained (control), stained for H-2K$^b$, pulsed with peptide and stained for H-2K$^b$/SL8, not pulsed with peptide and stained for H-2K$^b$/SL8 (control). In order to stain the H-2K$^b$ complexes, FITC-conjugated Y-3 antibody was used. This antibody is reported to bind to H-2K$^b$ and few others MHC class I mouse alloantigens. In order to stain the H-2K$^b$/SL8 complexes, FITC-conjugated 25-D1.16 antibody was used. This antibody is reported to give a fluorescent shift when the alloantigens are bounded to SL8 peptide. Antibodies were diluted 1:100 in FACS wash from the stocks, and samples were resuspended in 50 µl of the diluted solutions for 30 minutes at 4°C. The incubation was followed by two washes (453 g, 2 minutes), before moving the samples to the flow cytometer for analysis. Results are shown in Figure 4.16.
Figure 4.16 H-2Kb and H-2Kb/SL8 staining of various antigen presenting cells. The overlapped FITC histograms for the different cell populations are reported in the four top graphs. Unstained (red) and 25-D1.16 in the absence of SL8 presented on the MHC class I complex (green) were the controls. Y-3 (light blue) represents the relative amount of H-2Kb complexes. 25-D1.16 (orange) indicates the relative amounts of H-2Kb complexes conjugated to SL8 after incubation with the peptide. The bottom grouped-column graph sums up the MFI of the various histograms among the cell lines. The experiment has been repeated twice, but data are not shown due to the high variability of the absolute values in the different days; nevertheless, trends were consistent and match the findings from the sensitivity assays reported in Figure 4.15.

Despite K89 and FSDC being the cell lines carrying more H-2Kb complexes, the seemingly higher number of complexes conjugated to the peptide of FSDC could not be confirmed due to the control, that showed that the cell line gave a high fluorescence even in the absence of SL8; the experiment has been repeated twice, but data are not shown due to the high variability of the absolute values in the different days; nevertheless, trends were consistent.
and match the findings from the sensitivity assays reported in Figure 4.15. For these reasons, K89 was kept as the cell model of election for the following experiments.

After confirming that K89 was the best cell line to engage B3Z responses, the protocol for peptide loading has been further investigated. Specifically, the goal of the experiment was to find the best incubation time in presence of peptide to optimize the loading, and for how long would the cell line keep the epitope exposed on the cell surface after washing off the unbound SL8 and resuspending the sample in fresh media. 25D1.16 dye was used to stain aliquots of the K89 population at various times. The controls of the experiment were an unstained population of K89, and a K89 sample stained with 25D1.16 in absence of SL8 peptide. Different samples of the cell lines were pulsed with the epitope for 20-40-60-80 minutes, before washing off the unbound peptide and tagging the H-2Kβ/SL8 complexes (Figure 4.17, left); in parallel, other K89 samples were incubated with peptide for 1 hour, and after washing off the unbound SL8 they were resuspended in fresh media: their the H-2Kβ/SL8 complexes were tagged at 0-30-60-90-120 minutes after resuspension (Figure 4.17, right).

![Antigen presentation on K89 surface over time](image)

Figure 4.17 Antigen presentation on K89 surface over time. MFI was obtained after 25D1.16 staining, and 95% CI was calculated for each sample. In the left graph, the samples were incubated for different times (20-40-60-80 minutes) with SL8 before staining; in the right graph, all samples were incubated with the epitope for 1 hour, and signal was read at different times after washing off the unbound peptide (0-30-60-90-120 minutes). Controls: unstained K89 and stained K89 in absence of SL8 (first two columns of each graph).

The first experiment showed that peptide presentation could be observed already after 20 minutes of incubation, and that the loaded amount was quite stable up to 1-hour incubation. The signal almost doubled prolonging the incubation of other 20 minutes. The second experiment showed a similar increase in H-2Kβ/SL8 complexes past the hour of incubation. This set of samples was incubated with peptide for 60 minutes only, and the further increase was observed after removing the unbound peptide, and stopped after a total of 2 hours from
the initial addition of peptide. The secondary increase could be related to a different pathway in the epitope presentation, e.g. antigens that have been internalized and coupled to the MHC class I complexes in the ER before exposure onto the cell membrane (endogenous pathway) rather than directly binding the MHC class I alloantigens normally exposed on the cell surface (antigen cross-presentation). After the 2 hours post stimulation, in absence of fresh SL8 to bind, the peptide exposure faded exponentially. 120 minutes after removing the peptide in solution, the level of H-2K\(^b\)/SL8 complexes was slightly lower than the amount exposed after 20 minutes incubation. This fascinating mechanism was not further investigated, as it deviated from the final purpose of the project. However, this set of experiments provided insight into the time-window for antigen presentation in subsequent microfluidic experiments: the incubation for the loading of the peptide should last more than 20 minutes, and once the excess epitope is washed off, the experiment must be completed within two hours, time after which the level of peptide will lower under the initial concentration; optimally, experiments shall be run at the same time after the wash, to guarantee a consistent peptide presentation. It is worth reminding that exceeding the two hours after sample preparation would also affect the reliability of the calcium staining of lymphocytes.

### 4.4.3 Force dependence in APC-T cell contact

Among all literature studying pMHC:TCR interactions, few groups have considered the mechanical stresses at the T cell-APC interface. Finkel’s group proposed that the TCR acts as a mechanotransductor, translating mechanical forces into biochemical signals. TCR is a small molecule surrounded by a layer of large glycoprotein, and it wouldn’t be surprising if a mechanical force is needed to enhance the chance of pMHC-TCR interactions. The opinion paper reviews different experimental works proving how calcium fluctuations could not be captured in absence of applied forces, and they report that indeed few pN forces are sufficient to cause conformational changes in membrane proteins. They sum up saying that “mechanical forces leading to receptor deformation seems to be the only model that can address all three aspects of the T cell triggering puzzle: mechanism, specificity, and sensitivity”. Other groups investigated the mechanotransduction initiating the signalling cascade, and modulating the T cells behaviours at all stages of the cell-cell interactions using very specialized equipment such as Atomic Force Microscopy (AFM), optical tweezers and decorated substrates with different elastic modulus. A recent paper from Zhu’s group...
proves how “force prolongs lifetimes of single TCR-pMHC bonds for agonists (catch bonds) but shortens those for antagonists (slip bonds)”. Basically, the group states that an applied force will increase the power of antigen discrimination.

In accordance with the previous literature, in order to optimize the B3Z stimulation protocol using flow cytometry, and eventually capture the B3Z calcium fluctuations due to actual biological activation, the parameters of the centrifuge step (time and speed) used to induce the cell-cell contacts were changed to assess whether the observed lack of responses could be attributed to low forces acting on the cells during these interactions.

After B3Z calcium staining and co-incubation of K89 with 10 µM SL8 for 1 hour, the two populations were aliquoted in different tubes. The basal fluorescence signal of each B3Z sample was recorded before K89 addition. B3Z:K89 were mixed at a 1:1 cell numbers ratio, and K89 were resuspended in a 50 µl volume of media just before addition to the 200 µl sample of B3Z. Each sample containing the cells mixture was then spun down with different time-speed settings, before being resuspended with a pipette and put back on the rack to record the B3Z calcium signals for other 2.5 minutes. Centrifuge times were varied between 1 and 3 minutes, while the speeds ranged between 59 and 835 rcf (800-1400-2000-3000rpm on a 5415D Eppendorf centrifuge combined with rotor F-45-12-11). Results are reported in Figure 4.18, and indicate the percentages of activated B3Z gated on the FL-1 histogram of the single B3Z population after K89 addition. A similar set of experiments was conducted in literature228, although the focus of the paper was the T cell-APC contact duration rather than the force influence. The group used a co-centrifugation of 20-40-60s at 2000g to bring the cells into contact, anyhow such high speed could be detrimental for sensitive cell lines.
It is easy to spot how the number of activated cells increases by both prolonging the centrifugation time and increasing the spinning speed. This result confirms the findings reported in literature, suggesting that the force applied on the APC/T cells during cell-cell contact has a role in the signalling cascade. Differently from Figure 4.14, the histograms appear narrower, corroborating the previous idea that activations by static co-incubation in a 96-well plate were present but not synchronized, and that the centrifuge step helped both in the activations synchronization and in obtaining faster responses. Such a result was never proved with common lab equipment, and should be kept in mind also in the microfluidic experiments, where the forces acting on the cells could be tuned more precisely and have never been investigated in literature while always being present, especially in setups employing microfluidic traps\textsuperscript{193}. These findings highlight how droplets-based platforms fail

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Figure 4.18 FL-1 histograms of single B3Z after 1:1 addition of 10µM SL8 loaded K89 and spinning of the mixed population at different centrifugation speeds and times. Percentages of activators among the samples are indicated in the graphs.
both in controlling the exact time of cell-cell pairing, and in controlling/providing an initial force useful in the T cell activation.

On a side note, I found that our initial assumption that a larger population of doublets would be expected among a population of B3Z activated by contact with APCs was incorrect. Figure 4.19 shows that the pellet resuspension using a pipette is sufficient to break the T cell/APC bonds, and percentages fluctuations can be attributed to variances in the efficiency of the manual pellet resuspension.

![Figure 4.19 Doublets percentages after B3Z:K89 centrifugation and pipette disruption of the pellet, gated on the FSC-A:FSC-H graph. No correlation between the spinning settings and the cell couples were observed. The random fluctuations could be related to variances in the manual pipetting.](image)

**4.4.4 Peptide concentration influence in B3Z calcium response**

In this last study, the B3Z population has been stimulated with K89 pulsed with different concentrations of SL8 peptide. B3Z were resuspended at a $10^6$ cells/ml concentration and run at medium speed (35 µl/min on the BD Accuri™ C6) for a total of 2 minutes (1 minute before and 1 minutes after K89 addition, sample pelleting and resuspension). K89 were added at a 1:1 ratio in a 50 µl volume, after one-hour incubation with peptide and one wash to remove unbound SL8. Samples were spun down for 3 minutes at 2000 rpm on a 5415D Eppendorf centrifuge combined with rotor F-45-12-11, as these were found to be the best settings for B3Z activation.
Peptide dilutions were prepared in media from a 10 mM SL8 stock. K89 samples were incubated in solutions containing peptide concentrations spanning between 10 µM and 10 pM (always preparing a control of K89 not incubated with the epitope). This is in accordance with the concentrations used in the sensitivity assays, while previous experiments in our group only documented B3Z activation against K89 pulsed with the highest concentration of peptide (10 µM)\textsuperscript{210}.

Data were analysed on viable single B3Z as usual; on the Time : FL1 plot, B3Z were gated before and after stimulation, and the signal was normalized on the initial fluorescence level of the B3Z population as \((\text{MFI} - \text{MFI}_0)/\text{MFI}_0 \times 100\). Activators percentages among the B3Z gate after the stimulus were obtained setting a threshold on the FL1 histogram of the population. In Figure 4.20, representative data from the gating process and the obtained stimulations are shown.
Figure 4.20 Representative data of B3Z biological activations obtained by spinning down the population with SL8-pulsed K89 and recording the MFI shift in flow cytometry. The top 4 graphs on the left show the gating process on a B3Z population stimulated with K89 loaded with 10µM SL8; the bottom 4 graphs on the left show the same gates on a B3Z population exposed to K89 not loaded with peptide. In both cases, the fourth graph shows the FL1 histogram of the B3Z before (light blue) and after (red) mixing with K89. The graph on the right compares the FL1 histograms of the “after” gates (obtained in the same process) of B3Z populations exposed to K89 loaded with a titration of SL8 concentrations. Percentages of B3Z activators were obtained adding a threshold on these histograms.
Normalized data are shown in Figure 4.21, and are consistent with expectations. A higher concentration of SL8 presented on the K89 surface induces a greater normalized increase in fluorescence, that corresponds to a higher level of intracellular calcium. The red line in figure depicts the average between the replicas. Figure 4.22 shows the percentages of activations among the B3Z population against the various concentrations of presented peptide.

Figure 4.21 B3Z activations against K89 pulsed with different SL8 concentrations. Each data point represents a tested sample; red lines represent the average among the replicas (n = 3).

Figure 4.22 Mean and SD of the percentages of B3Z activated against K89 pulsed with different concentrations of SL8 peptide. NO represents the control, where B3Z were mixed with K89 not loaded with peptide (n = 3).
In this set of experiments, three aliquots were also prepared separately, by resuspending the B3Z samples in different buffers (full media, media alone and DPBS) before K89 addition and data analysis. This aimed to see whether the difference in the responses due to the extracellular calcium level could still be highlighted, as previously shown for ionomycin activations. As a reminder, full media is expected to have a calcium concentration of 0.681 – 0.725 mM, while media alone has an ion concentration of 0.42 mM and DPBS represented the calcium-free control. All populations were stimulated with K89 loaded with 10 nM SL8. Despite being single data points, both the %MFI increase and the percentage of activated cells seemed to follow expectations, giving a higher response in presence of higher extracellular calcium levels (see Figure 4.23). This experiment wasn’t repeated as the extracellular calcium influence was already shown with ionomycin, and data already gave a good reason for determining that microfluidic experiments would be run in culture media, to better represent the in vivo responses; although partial, the data were anyway reported for the sake of thoroughness.

![Figure 4.23 Percentage of normalized fluorescent increase (left) and percentage of B3Z activators (right) when the T cells were resuspended in buffers containing different calcium concentrations: full media (0.7 mM), media (0.4 mM) and DPBS (calcium-free).](image-url)
4.5 Conclusions

In this chapter, the cell model to be used in the study has been thoughtfully characterized.

- Responsive populations of B3Z have been selected using dilution subcloning, and sensitivity to low concentrations of epitope has been assessed throughout the project.

- T cell responses were recorded against ionomycin (calcium ionophore, tested between 50 ng/ml and 10 µg/ml), antiCD3 (co-stimulator, tested between 5 µg/ml and 10 µg/ml) and antigen presenting cells pulsed with peptide in several concentrations (spanning from 10 pM to 10 µM).

- Calcium recovery has been investigated after ionomycin stimulation, showing that it takes around 10-15 minutes to restore the basal level.

- Extracellular calcium influence on the entity of the responses and percentage of activators were assessed: the physiological peak of intracellular calcium was recorded against a 20 µg/ml antiCD3 stimulation, and an extra 40% signal increase after ionomycin addition showed an active intake of ions due to the ionophore in presence of growth media. When cells were resuspended into a calcium-free buffer (DPBS), they showed similar response to the first stimulation with ionomycin, while subsequent ionomycin stimuli failed to elicit any further response. Results suggested that microfluidic experiments should be run in culture media to better represent in-vivo conditions.

- More importantly, it was proved that the selected antigen presenting cells (K89) were the best candidate in eliciting B3Z responses, both eliciting higher responses in the sensitivity assays and for the seemingly higher number of p-MHC complexes presented onto their surface.

- It was proved that the best timing to assess biological activation falls within two hours from sample preparation, both to avoid degradation of the fluorescent probe over time and for the amount of peptide exposed on the APC surface, that exponentially decreases one hour after antigen removal from the solution.

- Force dependence in the T cell activations has been shown in an inexpensive and innovative way, proving one of the advantages of single cell microfluidic studies is the possibility to better control influential parameters (such as force).
- Data showed that under the right conditions, B3Z responses can be expected within 3 minutes after cell-cell contact is induced. This timeframe was then used for recordings of single cells in trapping arrays.

It is important to notice how the synchronization of the responses is hard to control on bulk populations (shown both with static sedimentation of the cells mixture in a 96-wells plate and using centrifugation), and that possible differences of biological responses of specific T cells to APCs cannot be assessed using flow cytometry, as the readouts are averages of the time response on the entire population. In the case of biological activation, moreover, the exact time of the calcium peak could not be evaluated, as the fluorescent shift was happening during the centrifugation time.
Chapter 5: Microwells array for single cells time profiling

This Chapter will introduce the device that has been used to trap single lymphocytes in a highly-packed array of microwells in order to investigate their individual calcium signals over time. Section 5.1 will explain the design criteria of the platform, include the cells size determination methods and the master fabrication protocol. Section 5.2 will detail the steps to cast the trapping devices and include the results of the single cells isolation. Different materials to cast the devices will be evaluated, as well as the trapping efficiencies using different cell lines. Section 5.3 will illustrate the main steps of the data analysis process, performed using customized plugins and functions in ImageJ and Matlab. Paragraph 5.4 will conclude the Chapter by showing single lymphocytes responses to the soluble stimulants previously introduced: antiCD3 and ionomycin.

5.1 Microwell array design and fabrication

A high-throughput array for single cells trapping has been previously designed in our laboratory\textsuperscript{210}. In this section, the design parameters and the fabrication method of the master (negative mold of the final device) will be reported; then the device casting procedure will be explained, together with a comparison between different materials that could be adopted. A study on single cell stimulations using the same soluble compounds previously tested in bulk population experiments (ionomycin and antiCD3 antibodies) will prove the reliability of the platform, before moving to the discussion on the optimized platform to study single T cell responses to APCs loaded with epitopes.

5.1.1 Cells sizes determination

Single cell trapping arrays that use microwell designs rely on size exclusion. The well needs to be designed in order to contain one cell only, so the cell line size and its variations need to be taken in account closely before drawing the mask required for the device fabrication. A microwell array design to contain B3Z has been previously optimized in our laboratory\textsuperscript{210}. Due to the importance of the well size in relation to the cell line average diameter, the cell lines sizes characterization was repeated prior to use of the old platform. Two methods were
used to determine the average diameter of the T cell population. The first measurements were obtained using LUNA™ Automated Cell Counter; the counting device gives the option to obtain various statistics on the cell population, such as viability percentages and average diameter. The reading is fast, and the same device has been used for the first characterization of the cell line in previous experiments; nevertheless, its accuracy is strongly biased by the focus plan and background of the image (as shown in Figure 5.1), and at times it takes several readings to get a robust statistic. Different B3Z populations were tested in 7 not consecutive days, giving an overall average diameter of 14.31 µm.

Figure 5.1 B3Z size characterization using LUNA™ Automated Cell Counter. The device automatically recognizes the trypan blue–stained cells and it quantifies their viability as well as their average diameter (bottom histogram). Some artefacts (zoomed image on the bottom left) can reduce the reliability of the readings.
The second method used to determine the B3Z diameter employed single cell microfluidic impedance cytometry. The experiment was kindly run by Fabrizio Siracusa (Morgan’s group, University of Southampton, UK) in triplicates. Impedance cytometry is a useful non-invasive technology that allows to monitor different cell properties, such as cell size, stiffness and deformability.229,230 Relying on flow focusing, all cells are aligned in a microfluidic channel and pass through an electric field generated by couples of electrodes; the signal (impedance) of each cell (event) is dependent on its properties, and is then transmitted to a software; flowing calibration beads of known sizes together with the cell population it is possible to correlate the impedance signal from a cell to its volume, and deduct its diameter. This method is more precise than deducting the cell size from a 2D microscopy image, and the reading is immediate from a custom software that allows to gate the calibration beads and cells populations on the modulus/phase graph of the electrical impedance, and gives a histogram of the cell size distribution as an output. The flow was set to 10 µl/min, and 5 µm and 7 µm calibration beads were used to build the calibration curve. The module of the electric impedance is proportional to the volume of the cell, hence its cubic root is proportional to the cell diameter; the proportionality factor can be determined from the calibration beads, that have known sizes. The total average resulted to be 12.84 ± 1.53 µm, slightly smaller but comparable to the average obtained from the automated cell counter.
Figure 5.2 B3Z size characterization using single cell microfluidic impedance cytometry. On the left, the top image represents the cubic root of the module (x-axis) and phase (y-axis) of the electric impedance, where it is possible to gate the beads populations and the cell population (red); the bottom image shows the histogram of the diameters of the populations, that are proportional to the cubic root of the impedance module, and can be calibrated knowing the beads sizes. On the right, the histogram obtained from 3 samples of B3Z cells, fitted with a Gaussian curve on GraphPad Prism.

The results are comparable with the ones previously reported\cite{ref}, hence the same mask was used for the device fabrication. The design consisted of an array of pillars with a diameter of 18 µm, that will make wells with the same cross-section. Wells of such dimensions can comfortably contain a single cell of a diameter ranging between 12.8 and 14.3 µm, and are too small to host doublets.

### 5.1.2 Master design and fabrication protocols

The master fabrication process has been explained in Chapter 3.5. The photolithography protocol requires a mask design, that will define which features will be illuminated during the UV exposure step. The mask consisted of 324 arrays, each containing 4200 pillars hexagonally packed, with a horizontal shift between two centres of 35 µm, and a resulting vertical shift between rows of 30.31 µm. Previous results (reported in Figure 5.3), showed...
that the optimal well height to trap high numbers of B3Z limiting the percentage of doublets ranged between 17 and 27 µm.

Recipe 1 from Table 3.1 was used to fabricate the masters. Figure 5.4 shows one of the masters, and two ways used to check the features heights.
Figure 5.4 SU8 master and pillars size measurement. The top picture shows an example of an SU8 master obtained with photolithography (100 µm scale bar). The insight picture represents some pillars scratched from one of the arrays of the master; this gives an idea of the pillars cross-section and height. The bottom pictures were taken from the profilometer in the cleanroom, and show the tip that is scanning the master surface to assess the features shapes, and an example of profile that can be obtained (right). If the master is correctly developed, the side walls of the features are vertical, and the bottom is flat.

5.2 **Microwell array seeding protocol optimization**

In this paragraph, the protocol to fabricate disposable microwell arrays and to seed them with single cells will be illustrated.
5.2.1 Array material and cells viability

To fabricate the trapping plates, 2% agarose solution was prepared (2g pure agarose in 100ml filtered DPBS) and melted until transparent. In order to avoid any interference with the external environment and the calcium signalling, EDTA-free, DPBS buffer was used to dissolve the agarose. It has to be noted anyhow that during the incubation required for the cell settling, it is expected that most of the DPBS will be substituted by the media in which cells are resuspended; starting from a buffer that doesn’t contain any calcium will facilitate the control of the experiment conditions, as the final concentration will be the same as the one of the media in the surroundings of the cells (see Figure 5.6).

The molten agarose was then let to cool down while stirring up to when the solution reached a temperature of approximately 50°C. When at the right temperature, the solution was poured on top of the master. A PMMA ring around the wafer was used as a holder, preventing any leakage of the gel. Agarose solution was then left at room temperature to cure. After cutting the edges with a scalpel, the agarose stamp was flipped onto a plastic support, and it was cut at the right sizes to fit the holder for the microscope stage. If not used immediately, the plates were put on plastic supports inside culture Petri dishes, wetted with PBS underneath to prevent the drying of the hydrogel and covered with aluminium foil. They were prepared fresh every day of experiment and left at room temperature until used. They could be stored in a fridge for several days, without losing the features definition if correctly wetted. A schematic of the replica molding process is reported in Figure 5.5.

![Figure 5.5 Replica molding of agarose microwell arrays. The steps to obtain a hydrogel trapping device are depicted in the figure. From left to right: agarose is dissolved in PBS, reaching temperatures of 80-90°C; the solution is then cooled down to 55°C on a magnetic stirrer and poured onto the device master. The hydrogel is let to solidify at room temperature; devices are peeled off from the master and cut into the desired shape using a scalpel.]
Figure 5.6 Calcium ion diffusion into agarose trapping plate. The simulation was run using “Transport of diluted species” Comsol package. Coefficient of diffusion of calcium into agarose was taken from 231; initial calcium concentration in the trapping plate cross-section was set to 0, while species influx concentration on the upper wall of the design was set to 0.7 mM (as expected to be for full media according to what discussed in 4.2.2). Left scale indicates the plate height (mm), while the colour legend shows the calcium concentration (mM) at each spot. The simulation was run over a 30-minute period, that corresponds to the incubation time allowed for cells settling. The top series of images analyses the full 5 mm cross-section of the gel, showing that in 30 minutes calcium ions can penetrate roughly 2 mm in the hydrogel; the bottom time series focuses on the top layer containing the 30 µm deep wells, and shows how cells will be surrounded by a calcium concentration that is high enough to allow activation (intracellular calcium concentration at peak of activation is around 0.5 µM232).
Different materials were tested in the casting process: 2% agarose, 1:10 PDMS and 12% polyacrylamide. In the case of PDMS, the 1:10 curing agent:elastomer mixture was mixed and degassed in a vacuum chamber before being poured onto the wafer previously heated to 100°C on a hot plate, and was left to cure for at least one hour. The procedure to fabricate patterned polyacrylamide slides will be discussed in 7.2.1. All fabrication methods gave high definition in the wells profiles (see Figure 5.7), and each material can be used for different goals.

Figure 5.7 Microwell arrays of different materials made using replica molding. From the top: trapping plate made with agarose 2%; device in PDMS 1:10; array in polyacrylamide 12%. Scale bars 100 µm.
For example, PDMS has the advantages of being biocompatible, transparent and widely used for chip fabrication, offering a wide literature support. It also offers the advantage of not being susceptible to swelling/drying not being a hydrogel, so it doesn’t require a controlled environment of operation. It can especially be used for closed geometries, for instance it could be used to entrap the microwell array in a closed chamber connected to microfluidic channels of input/output, and it is easily activable for sealing with other materials (e.g. glass) or for functionalization. Nevertheless, being highly hydrophobic per se lowers its wettability properties, and each device will require an extra treatment to achieve good occupancies of the single wells if using simple passive sedimentation. While being widely adopted in every microfluidic lab, it is not generally available for prompt use in biology laboratories.

Polyacrylamide, on the other hand, is widely used in every laboratory for its electrical conductivity properties. It is employed as a molecular sieve in gel electrophoresis, and the recent technology of single cell western blotting, that uses microwell arrays, made it appealing for this project as well (as further discussed in 7.2.1). Nevertheless, acrylamide prepolymer is toxic, and if working with live cells an extra step to wash off all the uncrosslinked monomers needs to be considered. In fact, when B3Z viability was tested after seeding onto this hydrogel, viability dropped to 5.7% after only one hour, and improved only after the gel was washed overnight in DI water. The experiment (and comparison with agarose gel) was conducted as follows.

In order to test the cell viability in contact with the gel, a blank slab (with no features imprinted) was prepared at 12% polyacrylamide concentration. The gel was then cut and put as a bottom layer into a small Petri dish. The same process was repeated casting a small layer of 2% agarose on the bottom of a second Petri. Six plates were prepared for each gel. $2 \times 10^6$ B3Z cells were added on top of each gel in the Petri dishes, each resuspended in 500 µl of full media; the media was topped up every 30 minutes throughout the experiment to keep the gels hydrated, trying to avoid to dilute the cells excessively, in order to keep them relatively close to the gels surfaces. The same experiment was then repeated with other six polyacrylamide plates. In this case, the gel was prepared the day before, washed twice with DI water (using a wash bottle), left in DI overnight and washed an extra 2 times before testing. In all experiments, cells viability was tested at time zero, after one hour and after two hours (by aspirating the cells from the Petri dishes containing gels at the said times). Cells viability was evaluated using trypan blue and LUNA Automatic Cell Counter. The test was done over a 2-hours period, being the expected time live B3Z would be trapped in the plates for analysis. Results are shown in Figure 5.8, and report a good viability of cells in the presence of fresh agarose or highly-washed polyacrylamide, while 90% of cell death was observed within one hour of exposure to polyacrylamide not effectively washed. It needs to
be reminded that these experiments were run on flat gels, hence in the context of single cells trapped within wells, the toxicity could be even higher.

To avoid extra steps, and for its properties, agarose proved to be the best material of choice: it is biocompatible, widespread among biology laboratories, permeable to nutrients, oxygen, calcium and stimulants, and its intrinsic wettability allows very high wells occupancies.

![Figure 5.8 B3Z viability in presence of different hydrogels. Three cell samples (from different plates) were tested at time zero, and after 1-2 hours of exposure to different hydrogels: 2% agarose, 12% polyacrylamide and 12% polyacrylamide repeatedly washed in DI water.](image)

**5.2.2 Cells seeding and stimulation protocol**

The trapping plate relies on passive sedimentation to capture single cells in the wells. The protocol to isolate the lymphocytes follows the steps shown in Figure 5.9. Agarose plates were cut in a 2*2 cm square; each plate roughly contained 24 arrays of 4200 wells each. Cells were fluorescently labelled (as reported in 3.3) and resuspended in 300 µl of full media for each plate; the cell suspension was pipetted onto the gel, taking care that the drop was confined on top of the plate. As reported in 4.4.1, cell sedimentation velocity was assumed to be 0.58 mm/min. 300 µl correspond to a volume of 300 mm³, and considering a surface of 400 mm² of the plate, the maximum distance between a cell and the agarose surface should be around 0.75 mm only. Theoretically, cells should sediment within less than 5 minutes inside the wells; nevertheless, both to allow calcium intake in the hydrogel volume and to give cells plenty of time to enter the traps, cells were always allowed to settle for 30 minutes in an incubator. Thereafter, excess cells were removed from the surface by repeatedly washing the agarose surface with PBS. The plate was tilted of roughly 30°, and a wash bottle was used. The saline was not sprayed perpendicular to the surface, but rather from the upper edge of the plate, and excess solution was drained with absorbing paper from the bottom.
edge of the gel. Plates were checked under a microscope, to make sure that there were no floating cells left onto the plate. Hence, the gel was ready to be brought to the microscope stage, and loaded onto the customized holder. Holders were fabricated in PMMA with a laser cutter.

Figure 5.9 Representation of the single cells trapping protocol. From the left, cells are labelled for intracellular calcium with Fluo8-AM; the cell suspension is seeded onto the agarose wells array and cells enter the wells via passive sedimentation; excess of cells that didn’t enter any well is washed off with PBS using a wash bottle, and the plate is moved onto the stage of the fluorescence microscope; stimulant is added and single cell responses are recorded using time series.

Once plates were brought to the microscope, it was assumed the surface of the agarose plate was hydrated, but that any excess of PBS on top was dried away. The microscope stage was regulated to focus on one of the arrays with a 5X magnification, and basal fluorescence of the cells was checked. Soluble stimulants (antiCD3 antibodies and ionomycin) were pipetted on top of the array, taking care not to touch the surface with the tip.

Since the responses to stimulations were recorded with fluorescence time courses in the 5 minutes following stimulation, it was considered that the stimulant concentration would not be diluted by diffusion in the bulk of the gel. Hence, stimulants were added at the final concentration. It is reported in a technical bulletin from Beckman Coulter\textsuperscript{233} that the average velocity of the water dispensed by a standard pipette is 0.2075 m/s (given a maximum velocity of 0.415 m/s and a fully developed parabolic profile). Considering that, not to interfere with the imaging, the pipette approaches the displayed spot almost horizontally (likely in a 15-40° range, as for patch-clamp setups), it can be estimated that the stimulant will reach all the cells in the field of view at the same time, and time zero can be approximated to the instant of pipetting. Assuming that the stimulant solution is homogeneous, the stimulation should be uniform across the field of view, and consistent among the cells population. This has been empirically confirmed by the experiments ran using ionomycin, in which the fluorescent spikes were synchronized and instantaneous. For each recording, 20 µl of soluble stimulant were added.
Another advantage of this platform is that, while being an open system, it is very versatile and compatible with a range of applications. In case the final goal of the study is to stimulate/stain cells with soluble reagents, without any further cells coupling, the gel can be easily integrated into a chamber having an inlet and an outlet; this would allow continuous monitoring while controlling the cells environment for longer times, and has great potential for automation of the entire experiment protocol. A rudimental proof of concept is shown in Figure 5.10, where two PMMA squares were laser cut to form the top and bottom layers of the chamber, and rastered to host an o-ring, that serves as a seal around the hydrogel, preventing any leakage. In this case, two pipette tips were used as simple connectors, and the chamber was tested for leakage over several days using food dye. No leakage was observed, thus demonstrating that, while other materials are more widely used for closed systems (such as PDMS or glass), hydrogel platforms can promptly be integrated in standard microfluidic systems.

![Figure 5.10 Integration of the agarose trapping plate in a close chamber. An agarose trapping plate was sealed in a chamber made of two PMMA layers using an o-ring. Inlet and outlet were connected to pipette tips, and the chamber was filled with food dye (left). The uniform diffusion of the dye in the bulk of the gel is shown in the right picture; the chamber showed no leakage over several days. Scale bar 1 cm.](image)

A second use of the agarose trapping device, not investigated in the present study, is drug testing. The advantage of working with hydrogel is that compounds can be diffused throughout the bulk in a controlled manner. Time-controlled drug release from a reservoir underneath the plate, or a combination of top flow and bottom diffusion are some of the possible applications.

On the other hand, one of the main disadvantages of working with hydrogel is the difficulty in washing off compounds from the bulk of the gel; this limits the possibility of studies where subsequent stimulations of cells are required, especially when working in short time scales. It has to be noted however that, while soluble reagents (ionomycin and antiCD3) have been tested on the present platform, to ensure that data were consistent with responses
observed in flow cytometry, further studies involving soluble reagents deviate from the goal of the project, and as such will not be further discussed.

5.2.3 Cells seeding protocol optimization

In order to find the optimal cells concentration to add to the plates to achieve the highest occupancies, a series of experiments has been run incubating different cell numbers on top of several agarose plates. All incubations lasted 30 minutes, and 2 plates were tested for each concentration. For each plate, multiple images of different arrays were taken and processed using ImageJ. The counts were run each on one entire spot of 4200 wells. Results are reported in Figure 5.11.

![Figure 5.11 B3Z occupancies using different cell:well seeding ratios.](image)

Good occupancies were observed starting from 5:1 cell:well ratio. Using cell concentrations of 10:1 or above, 90% occupancies were achieved. An excessive increase in the cell numbers resulted in greater chances of double occupancies (despite the limit given by the exclusion geometry), and washing out all the cells that didn’t settle inside the wells resulted more difficult. Floating cells are problematic as they can cross the field of view during the recordings, resulting in high interferences in the subsequent data analysis. For this reason, ratios higher than 10:1 were not investigated any further. Figure 5.12 gives an example of an agarose plate seeded with a B3Z population. As for the protocol, cells were previously calcium-stained; as such, they present a basal fluorescence level that can be detected (using a UV lamp and a FITC filter) also in absence of any stimulation.
While not directly relevant for the present study, in order to investigate the versatility of the platform, different cell lines having similar cell diameters have also been used to estimate their specific occupancies. The first cell line to be investigated was the fibroblast K89, since the initial setup to study the T cell-APC contact was designed as such both cell lines would be trapped in microwell arrays (see 6.1).
As for Figure 5.13, B3Z showed much higher occupancies percentages than K89, and this result was consistent with previous experiments\textsuperscript{210}. The two main hypothesis were that this phenomenon could either be related to cell sizes or to cell type, being B3Z suspension and K89 an adherent cell line. To evaluate which factor had more influence, K89 size was determined using the same methods previously adopted for B3Z, and described in 5.1.1: LUNA Automated Cell Counter and single cell microfluidic impedance cytometry (experiment kindly ran by Fabrizio Siracusa, Morgan’s group, University of Southampton, UK). Results suggest that the cell size shouldn’t be a major problem, as the measured average diameter is very close to the one of B3Z (12.47 µm according to the microfluidic impedance cytometry measurement, and 12.8 µm according to the LUNA counter). Even incubating the cells on ice to enhance their spherical shape didn’t help to increase the occupancies.

In order to investigate whether the cell type had an influence, a seeding experiment was also run using the alternative APCs that were introduced in 4.4.2. The results showed that high occupancies were obtained for the thymoma cell line EL4, suspension cell type, while melanoma cell line B16 and myeloid FSDC (adherent cell lines) showed very low occupancies. The cell lines average diameters (determined with LUNA Automated Cell Counter) were respectively 17.8 µm, 21.3 µm, 15.9 µm, proving that the size didn’t influence the loading efficiency. Occupancies percentages were found to be: 78% (EL4), 5.5% (B16) and 17.2% (FSDC). Although not being conclusive results, the likelihood of the cells to settle into the wells appeared to be related to their type. A possible explanation could be that adherent cells tend to form cell clumps due to cell-cell adhesion, and cells in the wells are more likely to be washed away if they created a junction with other cells during the settling incubation. Anyhow, it has to be reminded that thanks to the highly packed design of the
wells, even having wells occupancies lower than 50% would ensure a high-throughput screening of a relatively large population of single cells in a single field of view.

5.3 Single Cell Data Analysis

This paragraph will introduce the customized protocol to automatically identify single cells and study their responses. The present data analysis protocol relies on two softwares, ImageJ and Matlab. “Image Stabilizer” and “Time Series Analyzer” plugins needed to be installed in ImageJ to perform the analysis, together with a bespoke macro that defines the cells Region of Interests (ROIs). All the other scripts and functions were written on Matlab codes.

Data were recorded in the format of time series at the upright fluorescence microscope Zeiss AxioImager M2m; FITC channel was used to capture the Fluo8-AM fluorescence at a frame rate of 2 fps, for periods of 2-5 minutes and, when possible, keeping a fixed exposure time (usually around 100 ms). The concept of this protocol is shown in Figure 5.14: cells are automatically located in the plate from their fluorescent signal; the fluorescent intensity of each cell is plotted over time; lymphocytes that activated within the time window are selected, either automatically or manually; responders are located on the plate from their coordinates. This software allows to identify the single cells of interest within less than half an hour, and allows quantitative measurements on cell signals, while giving the potential to combine the study with other imaging techniques, since the cells are trapped in confined positions.

Figure 5.14 Representation of the steps for single cell responses analysis. From left to right: cells are automatically identified by their basal fluorescence against the background, and each single cell is assigned a circular ROI and a label containing its X-Y position; time responses of the cells are plotted and post processed to correct the background and dye degradation, and normalize on the initial fluorescence of each ROI; responders are selected, either automatically using algorithms or manually; responders are located onto the plate, retrieving their positions from their labels.
5.3.1 Regions of Interest (ROIs) definition

The time series was firstly opened in ImageJ, where images were stabilized using the Image Stabilizer Tool, that estimates the geometrical transformation needed to best align each frame to the previous one. This step is crucial, as the Regions of Interest (ROIs) drawn around the cells will create a mask with fixed positions, that will be used to record the greyscale values of the inscribed cells throughout the recording; if the images of the plate are shifted, this will result in processing values from the background, biasing the analysis with artefacts. Inconsistent frames at the beginning of the recording were removed from the recording (either when opening the files or using Slice Remover plugin): all signals are normalized on the first frame that needs to be consistent with the positions of the rest of the recording.

Thanks to the cells basal fluorescence and generally good contrast over the background, single cells could be automatically picked with the ImageJ function Find Maxima. Results were saved in a .csv file as a list of X-Y coordinates.

Circular ROIs were drawn around each maximum using a personalized macro, adapted from DesignROI macro, given an arbitrary number of pixels as a diameter (having a 5X objective, 14 pixel seemed to generally fit B3Z cells); ROIs were automatically added to the ROIs Manager Tool.

Time Series Analyser was used to extract the average intensity of each ROI (cell) over time, giving as an output an Excel file having the cells as rows and the time frames as columns. The first column of the table contains the cell reference, in the format of a string including the X and Y coordinates of the centre of the ROI. The table format is pictured below:

<table>
<thead>
<tr>
<th></th>
<th>T₀</th>
<th>T₁</th>
<th>Tₘ</th>
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</thead>
<tbody>
<tr>
<td>001</td>
<td>X₁-Y₁</td>
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<td></td>
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<tr>
<td>002</td>
<td>X₂-Y₂</td>
<td></td>
<td></td>
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<tr>
<td>n</td>
<td>Xₙ-Yₙ</td>
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<td></td>
</tr>
</tbody>
</table>

The Excel table was automatically saved and opened in Matlab, together with the list of the maxima, containing the coordinates of each cell.

In Figure 5.15, an example of automatic location of the cells within a trapping plate is presented. ImageJ uses a time frame from the recorded video as the top picture; by the personalized protocol, it is possible to build ROIs around each single cell in a simple and effective way. To prove the correct functioning of the “gating” process, the ROIs are overlaid
in yellow on top of the second image, that depicts the same plate in a composite image. In this case, cells were stained in red using a membrane staining, and it is possible to notice the solid correlation between automatically selected ROIs and well positions.

Figure 5.15 Automatic cell location and labelling. The same image is reported above: on the top, the greyscale image taken from the fluorescent channel, used to find the maxima and define the ROIs; on the bottom, the composite image (fluorescent channel and brightfield) is used to prove the correct ROI gating: the yellow circles are the ROIs, each of them is labelled with a number and the X-Y coordinates of its centre. Scale bar 100 µm.
A different software was previously developed in our lab for this task, where ROIs were selected based on the mask geometry. The big disadvantage of this program was that the ROI selection was performed based on the brightfield image. The selection could be done automatically (converting the picture to greyscale and using threshold and erode/dilate functions to enhance the wells contrast from their edges), or manually, knowing the wells diameters and interspaces: it was possible to pick the corner wells from the brightfield image, and the program would automatically create an array of squares that would surround all the wells in the plate. Following, wells containing cells were selected, and empty ones discarded based on visual inspection. The method was highly time consuming, and the selection was at times arbitrary. In addition, signals were analysed on squared ROIs, intrinsically carrying a background interference throughout the analysis.

The present code has three main advantages over the previous protocol: it is less time consuming (dealing with thousands of cells at a time, the ROI selection time was cut from hours to minutes), it is more reliable (directly picking the cell locations from cell signals) and it doesn’t depend on the mask design, making it easily transferrable between platforms.

### 5.3.2 Cells Selection

Once cell labels (number and coordinates) have been saved in Matlab, the code automatically processes the single cell signals. Raw signals are read from the Excel table imported from ImageJ, and plotted as a MFI:Time (Y:X) graph. This figure is compared to the average MFI signal of the population, inclusive of standard deviation. A scatter density plot is also automatically generated, and carries the same amount of information of a FACS recording, identifying each value in the time-traces table as an event on the screen. While giving an idea of the overall behaviour of the population, it lacks the ability of following the signal of a single cell over time. These three plots are reported in Figure 5.16.
Figure 5.16 Raw MFI signals of single lymphocytes over time. These MFI-Time plots represent the responses of thousands of B3Z isolated in an agarose trapping plate after stimulation with antiCD3 (time point: 10 s, black arrow) and with ionomycin (time point: 170 s, red arrow). While the top graph shows all the single responses as different lines, on the bottom the average response of the population is reported (left) together with their scatter density plot (right). The bottom graphs include the same amount of information carried on a FACS recording. From these plots, the percentage of B3Z that responded to antiCD3 stimulation seems to be 8.8%.

Hence, the signal is normalized in the same manner that was used for the bulk population signal in flow cytometry: \((\text{MFI} - \text{MFI}_0)/\text{MFI}_0\), where \(\text{MFI}_0\) is the value of the cell in the first frame of the recording. A second plot contains both the normalized signal of each single cell and the average of the normalized signal of the cell population, comprehensive of the standard deviation. A new scatter density plot is also automatically generated. Thanks to the normalization of individual cells, it is possible to “recover” traces of responders that would be classified as not activators in FACS analysis, due to the intrinsic lower fluorescence of a fraction of cells in the population. This can be observed in Figure 5.17.
Figure 5.17 MFI signals of single lymphocytes over time normalized on the initial fluorescent value of each cell. These graphs were produced from the same dataset used in Figure 5.16, where B3Z were stimulated using antiCD3 (time point: 10 s, black arrow) and ionomycin (time point: 170 s, red arrow). While the top graph shows all the single responses as different lines, on the bottom the average response of the population is reported (left) together with their scatter density plot (right). From these plots, the percentage of B3Z that responded to antiCD3 stimulation appears to be 19.7%. Without normalization, only 8.8% of responders could be gated.

At this point, the normalized data can be processed to automatically correct the overall fluorescence loss, likely due to photobleaching and/or dye degradation inside the cell. This is done picking the signal of a non-responsive cell as a reference: being a normalized signal, in absence of degradation, the fluorescence over time should be constant at 0; nevertheless, the intensity decreases. Hence, for each data point of the reference plot the difference between 0 and the actual value is calculated, and added to each signal from the other cells. As a result, all nonresponsive cells should have a corrected signal constant at 0, while the fluctuations in other signals should be directly correlated with the calcium signals, and the signal loss due to photobleaching artefact should be removed. Corrected normalized signals of all the cells are plotted in a new window, and compared with the normalized signals before correction (Figure 5.18). It is important to notice that some stimulations, such as ionomycin, can elicit 100% responses over the population. In such events, not having a non-responder signal, this step should not be applied. Although having a reference within the same recording is more accurate, in these cases is advisable to repeat a recording on another
trapping plate of cells not being stimulated, using the same settings as before. The average signal of the population in absence of stimulations can also be used as a reference signal.

![Graph of MFI signals](image)

Figure 5.18 Normalized MFI signals of single lymphocytes over time before (top) and after (bottom) photobleaching correction. These graphs were produced from the same dataset used in Figure 5.16, where B3Z were stimulated using antiCD3 (time point: 10 s, black arrow) and ionomycin (time point: 170 s, red arrow). In both graphs, all the single cell responses are reported as different lines.

At this stage, it is possible to pick the responders. This can be achieved in three possible ways, two based on automatic algorithms and one that involves manual selection.

As for the automated systems, the first is a threshold method that identifies responders as cells having signals that, at any point in the recording, scored a fluorescence value higher than an arbitrarily selected threshold.

The second method focuses instead on the signal slope: it requires as input the intensity shift of interest and the number of frames over which the shift should take place; all cells having slopes in their signal greater than the one given as an input are classified as responders. This
can be useful when the fluctuations are visually present but small and hidden in the background oscillations.

Third, the program gives the opportunity of manually select the signals of interest: it will plot all the signals in a table of different subplots, and the user can click on the relevant ones (that will turn red). While being more time consuming, this tool allows to promptly identify trends in the responses and to process signals that would be missed with the two automatic algorithms. It has to be noted that this method can suffer from selection bias and clustering illusions, due to the subjectivity of the selection.

In all cases, responders are automatically traced back by the program, which will extrapolate their positions on the plate from the list containing the coordinates of their centre. The responders table can be opened in ImageJ, and running once more the macro to draw the ROIs around the given coordinates, activators will be highlighted. These ROIs can be overlaid to any image, and could be used to facilitate any further analysis of the cells on the plate, or they could work as a guidance if responders are to be picked using micromanipulators.
Figure 5.19 Example of manual selection of the single cells of interest (top) and location of the responders on the trapping plate (bottom). Single cell responses are automatically plotted, normalized and corrected; afterwards, it is possible either to select responders automatically, using two possible algorithms, or manually (as represented in the top image), by visualizing sequentially all the single time signals and clicking on the ones of interest (red signals are the selected ones). Following, the program retrieves the information on the selected cells, and it relocates them on the plate by their X-Y coordinates. Scale bar 100 µm.

5.4 Single Cell Activations

In this chapter, lymphocytes activations were analysed with the described protocol, in order to prove the feasibility of the study at a single cell level using a fluorescence microscope. It is important to note that sensitivity and resolution of the two acquisition systems (flow cytometry or microscopy) are very different, preventing any direct comparison.

To help visualize the difference between the two methods (flow and microscopy): a sample of Rainbow calibration particles (559123, BD Biosciences), containing a mixture of 8 subpopulations of beads with different fluorescent peaks was analysed both using BD Accuri™ C6 flow cytometer, and with the Zeiss AxioImager M2m confocal microscope (using a 5X objective, a HBO mercury lamp and a FITC filter). Results are shown in Figure
While all the subpopulations were very distinguishable using flow cytometry, only 50% of the beads could be correctly analysed for each exposure time using microscopy. Also, while images in the time series acquisition need to be in 8-bit format for the post processing (meaning 256 possible grey values), the MFI shift observed between the basal state of a B3Z and its fluorescent level when activated was in the order of $3.4\times10^6$.

The lower resolution in microscopy analysis makes the detection of small calcium fluctuations very challenging. For this reason, while big fluorescence signals should still be observable (such as the calcium increase consequent to a lymphocyte activation), smaller oscillations will be lost in the same recording (i.e. keeping the same recording parameters).

Figure 5.20 Comparison between flow cytometry and fluorescence microscopy in the analysis of a sample of calibration particles having 8 different fluorescent peaks. All subpopulations can be observed simultaneously with the flow cytometer (top images), while only a fraction of the mixed population can be analysed using the microscope at a fixed exposure time. Sensitivity and resolution of the two systems are very different and prevent any direct comparison of the data. Time series images are 8-bit, giving 256 possible gray values, while the MFI shift observed after B3Z activation using flow cytometry was in the range of $3.4\times10^6$ MFI.
5.4.1 Ionomycin stimulation of single cells

The first experiment to prove the feasibility of the study was run using ionomycin. Fura2-labelled B3Z were isolated in agarose trapping plates, the devices were loaded in the PMMA holder on the microscope stage and an array of microwells with a good loading of single B3Z was selected for the recording. Few seconds after starting the time series, 20 µl of ionomycin at the final desired concentration were added to the selected area, taking care of not touching the plate while pipetting. The entire recording lasted approximately 5 minutes. The time series were then analysed as for the described protocol. It has to be noted that at times the focus changed after addition of the drop of stimulant. As such, Slice Remover (an ImageJ plugin) was used to cut the initial frames with a focus not consistent with the rest of the recording. For this reason, in all the videos it can be assumed that the stimulant was added exactly at time zero.

As previously mentioned, due to the mechanism of ionomycin, that acts both actively carrying calcium ions through the cell membrane and triggering a depletion of the internal calcium stores, 100% of the population responds to this kind of stimulation (when the ionophore concentration is high enough). As such, to compare the responses obtained with different concentrations of ionophore, the average time response across the entire B3Z plate was analysed. Four different concentrations of ionomycin were used, keeping the same parameters across the recordings (2 fps, 100ms exposure time): 100 µM, 10 µM, 100 nM and 10 nM. It was previously shown on flow cytometry that B3Z would respond to ionomycin stimulation in a range from 134 nM (100 ng/ml) to 13.4 µM (10 µg/ml). Results are reported in Figure 5.21.
Figure 5.21 Average over the responses of single B3Z to ionomycin stimulations. Four different ionomycin concentrations were used on different trapping plates, and the recording parameters were kept constant (2 fps, 100 ms exposure time). From the top: raw signals; signals normalized on the first frame; signals corrected using 10 nM trace as a “non-responder” reference signal.
The top graph shows the raw average time signals, while the normalized data are reported in the second graph. It is noticeable that the lowest ionomycin concentration (10 nM) is too low to elicit a response over the population. Indeed, when a similar concentration was used in flow cytometry (10 ng/ml, 13.4 nM), only 26.69% of the cells seemed to respond to the stimulation (compared to the 73.86% when using 100 ng/ml). Also, all signals present a similar fluorescent decay over time a part from 100 nM. This might be due to slower (and not synchronized) responses, that occur with time when more ionomycin molecules sediment in the wells and can be uptaken by the cells. For this reason, all the following experiments were ran using 1 µM ionomycin concentration, or higher.

The fluorescent shift due to ionomycin activation was also analysed using the slope method previously introduced: percentages of responders to the various concentrations of stimulant were quantified changing the two input parameters of the algorithm: the number of frames over which the shift would take place, and the entity of the shift. Results are reported in Figure 5.22 and Figure 5.23. All responders to the highest ionomycin concentration (93.3% of the population) showed a normalized fluorescent shift greater than 15 percent over 5 frames (2.5 s), while just lower percentages of the cells reported peaks with a similar entity in the same 2.5 s window when the stimulant concentration was lower. Adding 10 µM ionomycin, for example, only 1.53% of the population would be considered as responder if gating on events with 15 percent fluorescent shift; anyhow, when lowering the threshold to 5% increase over 2.5 s, it is possible to notice that 64.24% of the B3Z actually activated.
Figure 5.22 Percentages of B3Z responders to ionomycin stimulation according to the automated slope algorithm obtained varying the entity of the fluorescent shift over a 5-frames window. From the top left, % B3Z responders to 100 µM, 10 µM, 100 nM and 10 nM ionomycin. While all B3Z peaked in the 2.5 s window using 100 µM ionomycin, the signal slope was less sharp for lower ionophore concentrations.

Although all responders eventually peaked to a level at least 15% higher than the basal fluorescence, the time to reach the peak varies considerably depending on the stimulant concentration (see Figure 5.23). When considering the 100 µM stimulation, most cells peaked within 2.5 seconds, and the signal lowered quite soon. In fact, considering the cells with a signal 15% higher than the basal fluorescent over 10-15 frames (5-7.5 s), only 58% of the B3Z would be gated as responders. Instead, when using lower concentrations of ionomycin, most cells take longer times (7.5 s) to reach the same fluorescent shift.

All these considerations highlight how, while the amount of data that can be obtained using microscopy rather than flow cytometry analysis is much more complete and allows an higher level of analysis (e.g. the possibility to track single cells over time),
it becomes trivial to define algorithms that can fit all recordings to actually quantify the responders, and to classify the different signal patterns.

5.4.2 AntiCD3 and ionomycin stimulation of single cells

After proving the feasibility of the study, a new experiment was run to show the B3Z activations using subsequent stimulations of antiCD3 and ionomycin. As for the previous set of experiments, Fluo8-AM loaded B3Z were isolated in agarose trapping plates, the devices were loaded in the PMMA holder on the microscope stage and an array of microwells with a good loading of single B3Z was selected for the recording. Few seconds after starting the time series, 20 µl of antiCD3 were added to the selected area. 50 µg/ml and 100 µg/ml of antiCD3 were used alternatively in the recordings, and no major differences in the responses to the two concentrations could be noticed. After approximately 1.5 – 3 minutes (as specified in each experiment), 20 µl of 1 µM ionomycin were added to the same
spot, and the recording was continued for another 1.5 minutes. The time series were then analysed using the described protocol.

Some typical activation signals were plotted using GraphPad Prism 7, and can be found in Figure 5.24 (top). In this experiment, Slice Remover was used to cut the initial frames with a focus not consistent with the rest of the recording, hence antiCD3 addition coincides with time zero. It is possible to notice that the calcium increase due to antiCD3 activation presents a delay of approximately one minute, and is consistent with the results obtained using flow cytometry. When using FACS, the delay appears to be shorter (30 s), but it has to be remembered that, while the recording using microscopy is continuous, when running the sample on the flow cytometer the device needs to be paused, the sample is removed to add the stimulant and by the time it is put back and the machine starts to record again, the first 30 seconds from the addition of the stimulant are lost. This is also the reason why the signal increase due to ionomycin stimulation can’t be recorded using flow cytometry, being a sudden fluorescent shift.

Figure 5.24 (bottom) presents a correlation analysis between the MFI peaks of responders activated after antiCD3 stimulation followed by ionomycin around 50s later. There is a weak correlation between the maxima values measured following antiCD3 stimulation and the ones after ionomycin stimulation. When selecting double-responders, I noticed that in some cells where the calcium level had not returned to baseline at the time of ionomycin addition, a second peak could not be distinguished (e.g. black trace in figure).
Figure 5.24 An example of single B3Z responses to subsequent stimulation with antiCD3 and ionomycin. AntiCD3 was added at the beginning of the recording, while ionomycin was added approximately 100 seconds from the start of the traces. The top figure represents a random but representative selection of traces from double-responders (cells that presented a fluorescence peak after both stimulations); it is possible to notice the delay in the antiCD3 responses, that is consistent with the data obtained from flow cytometry. In the bottom plot, the full dataset of double-responders (n=155) was analysed for correlations between the MFI peaks following antiCD3 stimulation (timeframe between 40 s and 90 s) and MFI peaks following ionomycin addition (timeframe between 100 s and 140 s). Using the correlation analysis from GraphPad Prism 7, the two peaks seemed to be partially related (Pearson correlation coefficient 0.37), influencing each other by 14% (R² value of 0.14). The small P-value confirmed the validity of the analysis.

In Figure 5.25, responders from three independent experiments were manually selected using the usual protocol, and a heatmap representing their normalized signals was generated using Matlab. Each row of the plot represents the time response of a single activator. The same dataset is also depicted in Figure 5.26, where the colormaps have been adjusted to increase the resolution of the activations. According to the manual selection, 22.6% (183
cells out of 809), 13% (101 cells out of 782) and 15% (128 cells out of 855) of the B3Z populations were flagged as responders. Nevertheless, as already mentioned for the responses to ionomycin, the threshold to consider a single cell as an activator is arbitrary (reason why rather than setting a cut-off, signals were here picked manually, based on morphology). It was also observed that, while only a fraction of the population responded to antiCD3, the activation subsequent to ionomycin was ubiquitous, as expected.

Figure 5.27 represents the time signals of the entire population that was analysed in Figure 5.25 and Figure 5.26 (first plot of both images). In Figure 5.27, the cells (rows) are sorted based on their average value over the window between 30s and 180s.

When visually selecting the responders, only the signals with a clear and sharp slope leading edge, a defined peak and a slower slope trailing edge were picked, resulting in a 22.6% activators detected. When observing the heatmap of the entire population though, a clear fluorescent increase can be noticed over a much larger percentage of the population. The reason why a great part of the (presumably) activators was not selected is the different pattern in the time signal: almost half of the cells reported a slow and constant calcium increase rather than a sudden shift in fluorescence. These subpopulations could not be distinguished using flow cytometry, and illustrate the increased depth of information that can be achieved with continuous single-cell analysis.

Figure 5.28 and Figure 5.29 include an analysis of the traces reported in Figure 5.27. For each of the two subpopulations (fast and slow responders) correlation analysis was undertaken for key parameters of the signals. These were chosen to assess the correlation between a) the fraction of total calcium store released after stimulation with a-CD3 vs peak height; b) calcium released with anti-CD3 vs total calcium store; and c) calcium released with anti-CD3 vs peak height. While some parameters seem to influence each other, generally the correlation was poor for the parameters selected. Nevertheless, a significant difference was observed between fast and slow responders with respect to the correlation between calcium released with anti-CD3 vs total calcium store: that is to say the correlation was greater for fast responders compared to slow responders \( (r = 0.475 \text{ vs } 0.026) \). In general, these analyses illustrate the huge potential of data extrapolation from a single experiment using our high-throughput platform. This kind of post-processing could not be achieved with flow cytometry assays.
Figure 5.25 Heatmap of the normalized fluorescent signals (MFI-MFI₀)/MFI₀ of 3 independent sets of B3Z gated as responders to antiCD3 from a visual inspection of their individual traces. Each row represent the time signal of a different lymphocyte. AntiCD3 was added at the beginning of the recording (the artefact that can be noticed in the initial frames across the first and third sets was given by the pipette at the moment of stimulant addition); ionomycin was added after approximately 200 s, resulting in a sudden fluorescent increase.
Figure 5.26 Heatmap of the normalized fluorescent signals (MFI-MFI0)/MFI0 of 3 independent sets of B3Z gated as responders to antiCD3 from a visual inspection of their individual traces. Each row represent the time signal of a different lymphocyte. The same dataset used in Figure 5.25 are here reported. The colormaps were adjusted to emphasize the activation peaks of the responders.
Figure 5.27 Heatmap of the normalized fluorescent signals (MFI-MFI_0)/MFI_0 of the entire B3Z population from which responders were gated in Figure 5.25 and Figure 5.26 (first dataset on both images). Each row represent the time signal of a different lymphocyte, and the traces were sorted based on their average in the time window 30-180 s. It is noticeable that many more cells showed a fluorescent increase after antiCD3 stimulation compared to the 183 flagged as activators in the visual analysis, but they didn’t present a sharp peak at any point in their activations, biasing the selection. These different behaviours could not be highlighted using flow cytometry recordings.
Figure 5.28 Analysis of single cell responses to double stimulation with antiCD3 and ionomycin. Top left: correlation plot to evaluate if there is any relation between the fluorescence peaks (maxima) reached after the two stimulations; no correlation could be highlighted. Top right: two activation profiles were classified from the same dataset, manually selecting the traces belonging to the two subgroups: “fast-twitch” activators (blue) and “slow burners” (red); in this graph, the average signals of the two subpopulations are overlaid. Timeframes containing the responses to antiCD3 and to ionomycin are gated on this image, and were used for subsequent analysis (refer to Figure 5.29). Bottom: comparison of the two subpopulation signals; traces from slow responders are included in the left graph (and their average is overlaid in red), while signals from fast activators are included in the plot on the right (average in red).
Figure 5.29 Correlation between MFI signals after antiCD3 and after ionomycin stimulations. The two subpopulations of responders identified in Figure 5.28 are here distinguished as “fast responders” (top graphs) and “slow responders” (bottom images). The same three correlations analysis were plotted for both subpopulations. Pearson coefficients were obtained in GraphPad Prism 7, and are reported for each figure; the asterisk in the fifth graph indicates that the statistic was not considered as significant. Area Under Curve (AUC) were obtained from the timeframes gated in Figure 5.28 (bottom right image). From the left, the following correlations were evaluated: 1. Fraction of total Calcium mobilised by antiCD3 (= AUC for anti-CD3 spike / (AUC for antiCD3 + AUC for iono)) versus the peak in the antiCD3 response; 2. Calcium released by antiCD3 (AUC antiCD3) versus calcium released by ionomycin stimulations (AUC iono); 3. Total AUC for antiCD3 (AUC antiCD3) versus maximum reached during antiCD3 activation.
5.5 Conclusions

This Chapter introduced the setup and protocols developed to study single cell activations. Initially, the lymphocytes average size was evaluated using two different methods (LUNATM Automated Cell Counter and single cell microfluidic impedance cytometry). The measurements confirmed that the same mask previously designed in our laboratory could be used. The master fabrication protocol using photolithography and device fabrication protocol using replica molding technique were reported following. Different materials for the device fabrication were discussed, and B3Z viability data for each material was reported. A diffusion Comsol simulation proved that the environment surrounding the cells has the right calcium concentration at the recording time, after the incubation that allows cell loading. Hence, the cell seeding and stimulation protocols were detailed, and a set of experiments defined the best cells:well concentration when isolating the single lymphocytes. Well occupancies were evaluated using different cell lines, suggesting that suspension cells tend to settle more easily inside the wells, giving occupancies higher than 90%. Paragraph 5.3 detailed the single cell data analysis protocol, that is based on both standard and personalized ImageJ plugins and on a custom Matlab code. Specifically, the single cells were located on the screen based on the fluorescence maxima of a frame from the time series, and circular ROIs were drawn around the maxima coordinates. The MFI values of each ROI were obtained over the recording, and the single cell time traces were plotted and post processed in Matlab. Three algorithms (two automated and one manual) to rapidly identify the activators were proposed, allowing a prompt localization of all the responders on the trapping plate. The Chapter is completed by a set of experimental data obtained from single cell activations to soluble reagents (ionomycin and antiCD3), proving the feasibility of the study with the specific setup. Eventually, the need for new criteria to determine the activators was discussed, showing the large amount of new data that can be collected using the present platform compared to usual flow cytometry analysis: having access to thousands of time traces at a single cell level rather than observing an average behaviour it was possible to have access to new biological insight, such as the identification of subgroups of responders (fast-twitch, slow-burners) and a more precise quantification of the actual responders percentages (thanks to the normalization of the time traces on the basal calcium fluorescence, and the continuous observation of the cells over time, that might reach their activation peaks at different times. This system is highly adaptable, and the addition of different dyes could give insight in correlations between different biological parameters (e.g. signalling patterns and cell cycle). Finally, while heterogeneity could be highlighted already in a population of clone cells, the device would be used to its full potential when screening populations of intrinsically-different naïve lymphocytes.
Chapter 6: Cell-cell contact

In this Chapter, various options to bring the single T cells in contact with antigen presenting cells will be discussed. Initially, a magnetic coupling approach was attempted, but not pursued due to limitations which are discussed. An alternative pairing method is proposed in Paragraph 6.2, where T cells individually trapped in the agarose device will be coupled with antigen presenting cells grown confluent onto a flat surface. Integration of the device with a stage adapted from the rig used in Paragraph 6.1 will be detailed, together with controls that validate the setup and a more detailed analysis of experimental measurements of physiologically relevant T cell activation. Different protocols to pattern antigen presenting cells will then be discussed, and could be used in the future to refine the coupling mechanism. Paragraph 6.3 describes a microfluidic device that could be used to reproduce the obtained results onto a more transferable platform, detailing its design, fabrication, testing and limitations that would need attention in its future development.

6.1 Alignment and magnetic coupling

A previous setup to bring single lymphocytes into contact with antigen presenting cells has been previously proposed in our laboratory\textsuperscript{210}. A schematic of the flowchart is represented in Figure 6.1. The concept is to isolate both the populations of lymphocytes and antigen presenting cells in different trapping plates, then to oppose the “open” faces and align the wells to create multiple pairs of T cells and APCs. Ideally, the population of antigen presenting cells (bottom wells) would be loaded with superparamagnetic nanoparticles, deployed to “drag” APC out of the bottom wells in response to an applied magnetic field to create a simultaneous contact with the lymphocytes in the top plate. Individual T cell activation was measured using intracellular Calcium ion mobilisation as a readout as before.
Figure 6.1 Cell-cell contact using trapping plates alignment and magnetic coupling. From the top left: a. T cells (yellow) and APCs (purple) are isolated in two trapping plates; b, c. the devices are aligned to create multiple cells pairs; d. the magnetically loaded APCs are brought into contact with the lymphocytes using a magnetic field; e. time signals of the T cells are analysed; f. top responders are traced back into the plate.

Lymphocytes were loaded with Fluo-8AM, while APCs were loaded with superparamagnetic nanoparticles and pulsed with the antigenic peptide; the populations were loaded onto trapping plates using passive sedimentation protocol; the bottom plate (containing APCs) was mounted onto a 5-axis aligner, that was locked to the stage of the fluorescence microscope; the top plate was enclosed between PMMA holders, specifically designed to be coupled to the 5-axis aligner and to bring the two well plates to face each other.

The five-axis aligner was then used to match the wells positions of the two agarose devices (X-Y movements); the Z-axis controller was used to bring the plates into contact. At this point, it was assumed that all the cells would be at the bottom of the wells, and that the paired lymphocyte/APC would not touch each other due to their sizes (the cell diameter being smaller than the well height): it was suggested that leaving the T cells to settle in the wells for an extra 45 minutes after washing off the unsettled cells would prevent the lymphocytes from coming off the traps once the plate was inverted.
To induce a synchronized cell-cell contact, a 4kg pull-force block magnet was loaded onto the top plate holder, and left in position for 10 minutes.

A schematic of the setup is reported in Figure 6.2.

Figure 6.2 Schematic of the setup recreated with Solidworks. The 5-axis aligner (New Focus, 9082, Newport) was mounted onto a PMMA holder fitted for the microscope stage; on top of the aligner, a rotation stage (RP01, Thorlabs) was added, together with a holder for the bottom trapping plate (pink), containing the APCs. The upper trapping device (light blue) containing the lymphocytes was flipped on top, and clamped between two PMMA holders, that would lock it in position. The permanent magnet block was laid on the PMMA upper lid.

### 6.1.1 Superparamagnetic nanoparticle loading protocol

Superparamagnetic iron oxide nanoparticles were loaded into the antigen presenting cells (K89). These nanoparticles are widely used, and a robust literature indicates that they don’t alter cells viability or functions: they can be internalized via spontaneous endocytosis or phagocytosis, and will eventually be metabolized by the lysosomes. Sigma-Aldrich Iron Oxide(II,III) magnetic nanoparticle solution (10nm diameter, carboxylic acid functionalized, product no 747254) were used.

The loading protocol consists in an hour of co-incubation of cells and particles in media containing 1% of serum. The low percentage of FBS is required to minimise protein adsorption onto the spheres, that could interfere with the intake. K89 were loaded with the nano-beads and pulsed with antigenic peptide (either SL8 or SHL8) in one go; cells were resuspended to a concentration of 10^6 cells/ml, and superparamagnetic nanoparticles were
added at a final concentration of 0.5 mM together with the peptide (final 10µM). The sample was left in the incubator (37°C, 5% CO₂) for one hour, followed by a wash. K89 were then resuspended in full media and ready to be loaded onto the trapping plates.

Superparamagnetism means that the magnetic moment (M) of the nano-bead can rotate freely inside the particle. Lysosomes can carry several millions of these grains, making a labelled cell responsive to an inhomogeneous magnetic field, such as the one generated by a permanent magnet. More precisely, a magnetic moment will experience a magnetic force only in the presence of a magnetic field gradient (∇B), thus loaded cells are attracted to the region of maximum field.

Literature suggests that particle uptake is homogeneous within a cell population, depending on the cell type/size, culture conditions and other factors (i.e. incubation time and iron concentration). For example, it was demonstrated that T-lymphocytes can uptake 4.85*10⁵ ± 2.01*10⁵ nanobeads when incubated for 1 h at an iron molar concentration of 0.6 mM; when loaded, they can be moved using a magnetic field at velocities ranging between 2 – 8 µm/s.

It is important to obtain such information for this study, to be able to assess the theoretical time that the antigen presenting cells, trapped on the bottom plate of the described setup, would take to cover the distance to come into contact with the T cells. Moreover, it is also important to prove that nanoparticle uptake is universal and homogeneous over the cell population, in order to guarantee that this contact happens at the same time for all the cells pairs. Hence, assessing the nanoparticle uptake for the cell lines used will provide a timing confidence interval for the “contact time zero” event.

6.1.2 Superparamagnetic nanoparticle uptake

The first experiment aimed to prove the universal uptake of superparamagnetic nanoparticles over the entire cell population. Both the cell lines used in the model (K89 and B3Z) were tested, to assess any preferential configuration in the setup.

After loading the cells according to the usual protocol, an aliquot of each sample was transferred to a 1.5 ml Eppendorf; the aliquots were then put into contact with a block magnet (4 kg pull force) for 30 minutes, to allow the pellet of magnetically-loaded cell to form on the wall of the vials, in correspondence of the magnet. The supernatant was then discarded using a pipette, and retained cells were then resuspended in fresh media, and the incubation in presence of a magnet and washing steps were repeated a second time. The same procedure was repeated in parallel on control populations of unloaded K89 and B3Z. Cell concentration
in the samples was assessed before and after each wash using a haemocytometer and flow cytometry count (in two independent experiments).

Cell viability was also monitored over time, to confirm that the beads don’t show any toxic effect on the specific cell lines. A part from checking for viability while collecting the data for the described experiment, two separate populations of B3Z and K89 were loaded with nanoparticles according to the usual protocol, and put back into culture flasks to grow for 48 hours. According to literature\textsuperscript{234}, in fact, the beads should stay confined within the cells unless high stresses occur, and should eventually be metabolised by the cell without any alterations on cells functions or growth. The experiment confirmed that there was no observable toxic effect after the 48h on the two cell lines compared to normal culture conditions, according to growth rates and percentage of viable cells.

Results proved that the nanoparticles uptake was global over both cell lines, as shown in Figure 6.3: loaded cells kept similar concentrations after the magnetic washes, while unloaded cells (control populations) were mostly discarded following the same protocol. Data obtained from different methods (haemocytometer and flow cytometry count) were consistent, indicating that the nanoparticles uptake is indeed homogeneous. Loaded cells that were cultured for 48 hours proved to be viable and still responsive to magnetic gradients.

![Figure 6.3 Superparamagnetic nanoparticles uptake in B3Z and K89 cell lines. From the left, a schematic of the incubation aimed to separate magnetically-loaded cells and unloaded cells; during the “magnetic washes”, a pipette was used to remove the pellet on the bottom without disrupted the one on the side of the sample. The graphs represent the percentage of cells left in the vial after removing the pellet at the bottom: the first graph was obtained by the cell count read on the flow cytometer (one wash only); the graph on the right is based on counts from a haemocytometer, both after one or two magnetic washes. Samples loaded with nanoparticles (np) are “B3Z + np” and “K89 + np”; unloaded controls are “B3Z - np” and “K89 - np”.

\textsuperscript{135}
6.1.3 Magnetically-loaded cell velocity

The second validation of the system that aims to bring single cell pairs into contact using a magnetic field consists in the estimation of the cells velocity range when loaded with nanobeads in the presence of a magnetic gradient. This will provide an estimate of the time of cell-cell contact after magnet insertion.

In order to estimate cell velocity, single cell magnetophoresis was used, as described in literature. Magnetically-loaded cells were pipetted onto a glass slide positioned on a microscope stage; a block magnet was glued to the side of the glass slide; the objective was adjusted to focus on the cells at a distance of 7 mm from the magnet. A scale bar was overlaid to the pictures to have a distance reference to use in the data analysis, performed using ImageJ. Cells moving towards the magnet were recorded using a time series at a known frame rate. This setup allows detection of the average velocity of single cells crossing the field of view, with a lower limit of detection given by the cell sedimentation time on the microscope slide (around 2µm/s according to literature). The distance of 7 mm was chosen to resemble the relative positions of cells and magnet in the actual rig; the video processing was carried on using Mtrack Tool plugin of ImageJ. Selected cells were manually located in multiple subsequent frames of the recording, and the software would automatically extrapolate the covered distances, and knowing the frame rate it would obtain the cells average velocities. The velocity distributions of loaded B3Z and K89 are shown in Figure 6.5, and are in agreement with literature values. Few outliers showing extremely high velocities can be noticed in both populations; these results might be artefacts (e.g. errors in locating the cell in subsequent frames) that would drastically bias the mean velocity of the cell population. For this reason, median values and 25-75% percentiles were considered instead, and are reported in the following table.

<table>
<thead>
<tr>
<th></th>
<th>K89 (µm/s)</th>
<th>B3Z (µm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% Percentile</td>
<td>4.226</td>
<td>3.717</td>
</tr>
<tr>
<td>Median</td>
<td>6.109</td>
<td>6.895</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>11.87</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Considering that both cell populations have average diameters of 13-14 µm and that the wells are 27 µm deep, the maximum distance that should be covered by the cells on the bottom wells to come into contact with the paired ones should be roughly 28 µm (see Figure 6.4). If the velocity of a loaded cell ranges between 4 µm/s and 14 µm/s, it should take 2 –
7 s to the cells in the bottom wells to cover the distance. This variability is not relevant in the present study, where cells activations are analysed over longer periods of time (up to 5 minutes), but it gives a good estimate of the expected time of cell-cell contact, that can’t be visually assessed with this setup.

Figure 6.4 Schematic of a cell pair in the aligned trapping plates, and the cell-cell contact induced by a block magnet positioned on top of the plates. The maximum distance that should be covered by the cell in the bottom trap is 28 µm.
Figure 6.5 Velocity distribution of magnetically-loaded cells in presence of a magnetic gradient. From the top: a schematic of the single-cell magnetophoresis setup. The microscope stage is positioned to focus on the glass slide plane, at a distance of 7 mm from the magnetic block, the movement of single cells (represented by the red dot) is recorded. A frame from a video, with overlaid the cells trajectories that have been defined using Mtrack tool plugin of ImageJ. Velocity distribution of the two cell lines (B3Z and K89): the average velocity of each cell is reported as a dot in the graph; 25% percentile, median and 75% percentile are highlighted by the vertical bars.
6.1.4 Weaknesses of magnetically-induced cell-cell contact setup

Although the magnetically-induced cell-cell contact setup gave some promising preliminary results\textsuperscript{210}, it had some major limitations, that can be summarized as follows:

- a good alignment of the two agarose plates was crucial for the experiment outcome, but very difficult to obtain; unevenness of the surface and friction between the gels in the process usually gave a patchy alignment and an inhomogeneous contact of the plates.

- cells trapped in the top plate are free to “fall out” of the wells due to gravity.

- it is difficult to guarantee that all cells arrayed in the bottom device experience the same magnetic gradient, and impossible to prove such movement due to the setup configuration.

- the magnet used to create the field is in the light pathway, obscuring a view of the cells while it is in place. Experiments were conducted leaving the block on top of the plates for 10 minutes, removing it afterwards and hence positioning the rig back onto the microscope stage, to record cells activations. This way, the first and most relevant time-points during activation were lost.

In retrospect, it became difficult to correlate the activations that were recorded with the controlled pairing mechanism, as the cell-cell interactions could very well be related to B3Z sedimentation from the top plate to the bottom wells and in a similar way the lack of activations may be related to an overall magnetic force on some cells not being strong enough to overcome friction and steric impediments given by the wells in that plate position.

This setup was based on the experimental observation that, despite the agarose being an inert matrix that doesn’t offer cells proper binding sites, when the loaded plate was flipped, most of the cells would stay in the wells (against gravity). Given that B3Z are suspension cells, and as such they don’t need anchoring molecules, this might be attributable to various causes: surface tension when the top plate is not yet in contact with the bottom one; a higher concentration of proteins due to media evaporation during the setup preparation (e.g. fetuin contained in FBS, a protein mix that contains a wide range of growth and attachment factors, that could enable the formation of weak peptide bonds between the proteins contained in the matrix and the cells). Nevertheless, not all cells were observed to remain in the wells, making this assumption not always consistent. Moreover, the same forces would be experienced by the cells in the bottom plate, thus opposing the effect of the magnetic field.
In experiments, I showed that magnetically-loaded cells would not (consistently) come out of inverted wells. Figure 6.6 shows a schematic of two tested setups. Although some cells would be missing in the second observation, the great majority of the cells remained in the trapping sites.

The same experiment was repeated also having the plate in the usual orientation (wells facing up); in these experiment few cells, leftover of the washes, were on the surface of the gel and not in the wells. It was observed and recorded in a time series that, while the cells that remained on the flat gel surface were moving towards the magnet, the ones inside the wells didn’t show any visible displacement, and the initial and final frames showed the same occupancies within the wells. The same result was obtained in different days and after different incubation times of the cells within the wells.

![Figure 6.6 Schematic of the experiments run to assess the magnetic displacement of the cells in the wells. The plate was enclosed in a PMMA holder between two microscope slides; a magnet was put on top of the plate to attract the cells from the wells; experiments have been conducted both with the normal orientation (left) or flipping the setup to help the cells release with gravity (right).](image)

These results showed that the magnetic force acting on the cells is counterbalanced by greater force components (friction due to the contact between cells and wells, possible weak bonds and so on). Different trapping plates have been tested, showing that even lowering the height of the wells, while lowering the occupancies of the trapping sites, doesn’t help with the displacement of the cells. An example from one of these experiments is reported in Figure 6.7. In this case, the plate was inverted and left in this position for 20 minutes; afterwards, a 4kg magnet was inserted underneath for extra 10 minutes.

Due to the lack of repeatability and the impediment to observing early time points of activation, I decided to look for an alternative setup to induce synchronized and controllable cell interactions.
Figure 6.7 Before (yellow), after (blue) and overlay pictures of a plate of magnetically loaded B3Z. The plate has been flipped to have wells openings looking down, and incubated in this position for 20 minutes. Following, a 4kg pull force block magnet was inserted underneath to attract the cells outside the wells for an extra 10 minutes. The plate was eventually inverted to its original position and captured. Cells were not fluorescently labelled, hence these pictures have been converted to a binary image and coloured to help the view. Cell losses were manually counted, and represented the 28% of the population. The magnetic displacement of cells from the wells does not appear to be a consistent method, and some cells might have fallen out of the wells by sedimentation. Scale bar 100 µm.
6.2 Activation with monolayered APC growing on a flat surface

As mentioned in 5.1, wells in the plate were 27 µm deep, enough to contain one cell of 14 µm diameter, but slightly less of the height required to fit two cells when in their round shape. Also, the primary interest is testing single T cells, while the focus on single antigen presenting cells is not mandatory as long as the antigen presenting cell are loaded with the same epitope, and as long as a single T cell is in contact with any one APC at the time of activation.

As such, it was suggested that the lymphocytes isolated in the wells could be stimulated by capping the trapping device with a flat lid where APCs were grown confluent. The modified setup is represented in Figure 6.8. In this case, when the plates are brought together, the cell-cell contact will be immediate across the entire area, and won’t rely on tricky pairing mechanisms (like the magnetic displacement previously proposed). In fact, each antigen presenting cell positioned on top of a well, while still being attached to the slide, will protrude inside the well hosting a single lymphocyte, inducing a contact controlled by the geometry of the well.

Figure 6.8 Schematic of an alternative setup to pair single lymphocytes with APCs. The T cells (green) are isolated in an agarose trapping device (a), while the antigen presenting cells (red) are grown to confluence on a flat surface; such slide is then flipped onto the hydrogel plate, creating a control contact between the cell populations (b). c) shows an overlay of the two plates on top of each other, where T cells were labelled with Fluo8-AM and APCs were stained with PKH26 dye. Scale bar 100 µm.
There are two main advantages of this design over the previous setup, the first being that the cells are brought together in a simple and easily reproducible way: lymphocytes are isolated inside the wells facing up, not risking to be displaced from their positions, and antigen presenting cells are adhered to the surface by well-defined mechanisms, and these adhesion factors ensure that once the slide is flipped with the cells facing down, they won’t detach from the surface. The second main advantage is that, if the adhesion surface is transparent, it is possible to observe the very first moments of cell-cell contact, with a good redesign of the plate holders to fit the microscope stage. This was not possible previously, when the lymphocytes responses could be monitored only with a 10-minute delay and after removing the magnet from the light pathway.

As for the previous setup, for a good outcome of the experiments the agarose surface needs to be as flat as possible to guarantee a homogeneous contact between the planes; nevertheless, while a suboptimal surface profile would impede the alignment and cell-cell contact before, in this case cell pairing can generally be achieved in most cases.

The two main limitations of this setup are that the antigen presenting cells that will face the agarose surface rather than a position above a well may be displaced or undergo a stress-related response as they are compressed between the plates, and that there is no way to control whether the lymphocytes will be exposed to one or multiple APC. Although these drawbacks did not appear to interfere with the outcome of the experiments, it is possible to potentially overcome these issues by patterning the cells with an arrangement that mirrors the wells positions. Some considerations on cell patterning will be discussed later on in Paragraph 6.2.4.

6.2.1 Cell-cell contact using flat surfaces

Preliminary experiments to assess whether this setup could work were run by growing K89 on different flat surfaces (tissue culture Petri dishes, glass slides and fibronectin-functionalized PDMS membranes), pulsing them with SL8 while attached to the slides and flipping the slides on top of agarose trapping plates loaded with indicator-stained B3Z. A gentle pressure was then exerted with a finger to press the surfaces into contact, and the plates were then rapidly moved to the microscope stage, where lymphocytes calcium fluctuations were recorded.

Control recordings included time series of the same cell population of B3Z before capping the plate with the antigen presenting cell lid and time series of B3Z paired with the same flat surfaces in the absence of K89 and in presence of K89 not loaded with peptide. Results are
shown in Figure 6.9 and Figure 6.10. Using the custom script, fluorescence signals of single T cells were normalized on the initial value and expressed as a percentage of variation from frame zero. Heatmaps represent the single cells (individual rows) over time.

It is possible to see in Figure 6.9 that no significant calcium fluctuations were recorded in response to the mechanical operations involved in the device, and that both PDMS and tissue culture-grade plastic are appropriate surfaces to use. Importantly, T cells did not respond to monolayers of K89 APC grown on either surface (Figure 6.10, a). I then measured activation following exposure of arrayed T cells to K89 pulsed with 1 µM SINFEKL epitope. Figure 6.10 (b) clearly shows a population of responding T cells representing approximately 70% of the 1,200 individual cells measured, with a range of individual activation profiles.
Figure 6.9 Controls. a) single T cells signalling in absence of any stimulation (being in contact with the agarose trapping device only). b) single T cells responses when pushed against a PDMS surface. c) single T cell responses when pushed against a tissue-culture Petri dish. Traces were normalized on the first frame and expressed as a percentage of the initial value.
Figure 6.10 Signalling of single T cells paired to K89, in absence (a) or presence (b) of peptide. B3Z were trapped in agarose devices, while K89 (bare or pulsed with 1 µM SIINFEKL) were grown confluent onto Petri dishes. Cells were paired manually flipping the dish on top of the agarose plate, tapping the surface and recording signals subsequently at the microscope. T cells traces were normalized on the first frame and expressed as a percentage of the initial value.

To refine the analytical workflow, I investigated key parameters that I could use to differentiate “responders” from “non-responders”, and integrate these into the automatic selection protocol in the custom software. These included initial rate of calcium accumulation, and various threshold values. The optimal criterion was that cells would be automatically picked if intracellular calcium exceeded an arbitrary threshold (e.g. 100 in this case) within the first 65 frames (corresponding to 40 seconds). Their traces were overlaid and the average response was calculated, to allow comparison with other population-based measurements (e.g. flow cytometry). To confirm that the single-cell activation data are traceable, T cells with activation profiles that warranted further investigation (for example downstream processing for single cell recovery followed by RNAseq) were then identified on the plate (results shown in Figure 6.11)
Figure 6.11 Selection of the first activators against SL8-pulsed antigen presenting cells. From the top: heatmap of the activators (cells with signal greater than 100 within the first 40 seconds); responders’ traces over time and their average response (in red); identification of the activators on the trapping plate. Scale bar 100 µm.
From the heatmap in Figure 6.10 b, two kinds of responses seemed to appear: a population of early activators and a population of refractory responders. While the graph was cropped to match the timescale of the control recording, the two subgroups are better highlighted in Figure 6.12:

- The population of early activators has a rapid intracellular calcium accumulation beginning at around 30s after the start of measurement (around 120 seconds after cell-cell contact was initiated) and rises to a peak around 20s later, before slowly re-equilibrating;

- The population of refractory activators has a slow calcium accumulation beginning at around 80s and rising to a plateau (or broad peak) at 150s representing only around 25% of the maximum achieved in the first population.

The two populations were gated as follow. All the cell traces were initially sorted from the signal containing the highest peak value (sorted cell number 1) to the lowest. A subgroup of strong activators was analysed selecting sorted cells 1 to 100; a subgroup of low responders was compared to the first by studying the traces of sorted cells 700 to 800 that, while reaching only lower fluorescence values, could still be considered responders (from a visual inspection of the single traces): shifting the gate further in the sorted cells in fact proved to include non-responders in the analysis.
Figure 6.12 Comparison of two kind of T cell responses. a) shows the time of peak of all the responders, that were sorted based on their peak values (from the highest to the lowest). GraphPad Prism 7 was used to analyse the data and show the degree of correlation between peak value and time. Two subpopulations of responders (yellow and blue) were then selected and plotted in b) (yellow) and c) (blue). For both subgroups, the first graph shows in red the average response, while an heatmap of all the selected responses is shown below.

There was reasonably good correlation (Pearson correlation coefficient 0.64) between the time of peak calcium and the peak calcium value. The R² value of 0.41 suggests that the variables influence each other by 40% and the small P-value would confirm low chances that the correlation is a result of random sampling. Although the lowering of the peaks might be related to some degradation of the fluorescence signal after prolonged exposures, all signals were normalized and corrected over time. It has to be noted anyhow that the used...
automatic compensation for signal degradation is suboptimal. In fact, with the current analysis all signals are corrected based on the average degradation observed on the recording; nevertheless, after the correction is implemented, it is usually possible to observe a remaining variation between the non-responders’ signals: some of them drift to positive and others to negative values (instead of maintaining a flat trace over time). This suggests that the implemented correction for signal loss does not suit all the single traces.

This series of experiments showed the potential of the new cell-cell pairing system for measuring single T cell activation in high throughput format following physiologically relevant antigen stimulation (by live antigen presenting cells). Refinement of the instrumentation, to allow precise Z-axis control, to minimise variability inherent in manual coupling of the plates, and to enable the very first seconds of activation captured was the next focus of the project.

### 6.2.2 Integration of the pairing system in a microscope stage

New holders for the plates in the alignment rig were designed to integrate the new cell-cell contact system in a more consistent and controllable device. Moreover, while the activations obtained in the previous experiments could be recorded just after approximately 2 minutes from the cell-cell contacts (after moving back the plates to the microscope stage and focusing to the right plane), fitting new holders on the alignment rig previously used in the magnetic cell pairing setup allowed to employ the Z-axis of the stage to create the cell-cell contact while being already focused on the right spot, so that time zero could be properly captured. Experiments were run in triplicates at minimum, proving the functionality of the setup. The setup in shown in Figure 6.13 (a), together with fluorescence and brightfield images of the two cell lines. This setup allowed direct observation of the APC monolayer and confirmed its density and consistency within the device.
Figure 6.13 Integration of the pairing system in a customized rig. a) system mounted onto a fluorescence microscope stage. The 5-axis aligner and agarose trapping plate were maintained from the previous version of the pairing system, while new holders were designed and laser ablated to fit and hold in position a glass slide/PDMS membrane covered by APCs. b) example of the pairing of B3Z and K89. B3Z (green) are fluorescently labelled for intracellular calcium (Fluo8-AM), while K89 (red) are membrane stained (PKH26). On the bottom, two images of the same plates pair before (c) and after (d) the agarose plate is sealed against the glass slide using the Z-axis control of the aligner, while the focus was kept on the K89 level. It is possible to notice how the K89 in correspondence of a well-array will naturally be displaced on top of the wells when the contact is created, effectively coming into contact with the single T cells at the same time. Scale bar 100 µm.

Results from this kind of experiment were consistent, and similarly to the previous case, multiple recordings (n = 3) showed no response when T cells were paired to K89 not loaded with the antigenic peptide, while around 75% of the arrayed T cells responded when SIINFEKL peptide was loaded. Figure 6.14 shows some examples of traces obtained with the presented system.
Figure 6.14 Example of activation profiles from the integrated system. a) Time traces of the entire array of single cells. b) Time-resolved traces of the responders, sorted based on their mean values. c) Individual traces of selected activators (red signals) from an identical experiment.
Additional controls were run in this series of experiments to validate the measurements. First, K89 were membrane-stained using PKH26 dye, to enable their co-visualisation with the T cell array, thereby confirming that activation actually occurred only when T cells were contacting APC (Figure 6.15). The analysis was conducted as follows. An array of B3Z was selected and recorded over time using FITC filter to capture the signalling of lymphocytes. The same spot was subsequently recorded using Rhodamine filter to gate on K89 time traces. This showed that the membrane dye signal was constant over time, and did not induce artefacts in the FITC detection channel. Figure 6.15 (a) shows the K89 signals over time recorded with Rhodamine channel, proving that signals are mostly constant, and even in presence of a partial spillover of the signal over the green channel, no fluctuations would be recorded that could possibly bias the responders’ selection. Interestingly, the same ROIs (K89 positions) were overlaid to the FITC recording, and results are shown in Figure 6.15 (b). Some B3Z clearly share their well with antigen presenting cells, and their activation can easily be spotted. In both cases, a sample of individual traces was added underneath the graphs, to show some typical signal patterns. Using this method, 78 activated T cells could be identified. Similarly, B3Z ROIs were identified and used on the FITC recording (as for the usual analysis); according to this method, 86 responders could be highlighted. In this case, instead of tracing their position in one of the frames from the green time series, the selected activators were located using a picture from the red channel time series. Results are shown in Figure 6.15 (c), where the positions of the B3Z activators are circled in blue, while the K89 are coloured in yellow to help the visualization. It is clear that almost every activator indeed shared a well with at least one peptide-loaded antigen presenting cell. The discrepancy might easily be due to a slight misalignment of the cells in the same well, and subsequent signal loss in the processing. It also has to be noted that, while a total of 610 B3Z was counted in the analysis, the slight spillover from the PKH26 dye to the green channel might have altered the T cells selection. For this reason, statistics on the percentage of activators amongst the actual cell pairs would not be conclusive. In retrospect, a different dye with a further separation in the emission spectrum would help in the extraction of more data.
Figure 6.15 Combinatorial study of B3Z and K89 ROIs. a) Membrane-stained K89 signals in the red channel over time: no fluctuations could be detected (see examples of individual traces below). b) K89 ROIs were superimposed to the FITC recording of B3Z calcium signalling: 78 B3Z activations could be highlighted using K89 positions (see examples of individual traces below: examples of responders are highlighted in red). c) B3Z responders were detected from the FITC recording, and their position was highlighted with blue circles on a red channel frame (K89 were coloured in yellow using ImageJ to help the visualization): results match the finding pictured in b). Scale bar 100 µm.
As described in section 6.2.1, the presence of multiple subpopulations of T cells could be identified based on their activation profile. Figure 6.16 shows a comparison of single (a) and multiple (b) responders picked from the same recording, that was obtained pairing single B3Z to K89 pulsed with 1 µM SINFEEKL. As before, single responders’ activations were characterized by a unique and sharp initial peak followed by a slower recovery to baseline. For this group, the peak calcium intensity varied, and there was variation in the rate and profile (ie smooth or fluctuating). Differently from the earlier analysis, the second population of slower, lower and more sustained rise in intracellular calcium was not clearly identifiable from this set of experiments. Instead, in addition to the behaviours highlighted before, a third minor sub-population of activation profile was observed using the modified apparatus. This population was distinguished by multiple activation peaks. There was variation in this population also, but profiles typically showed 2-3 activation spikes with characteristics similar to sub-population (1), separated by around 100-150s. The two gated subpopulations are shown grouped in Figure 6.17 and in Figure 6.18, with their calculated average and examples of each subpopulation shown as individual traces. A correlation study to see whether the first responders also had the highest signals was conducted on both populations (single and multiple responders), but only a partial correlation in the multiple responders could be highlighted. The pulsatile behaviour has been described previously in the literature\textsuperscript{218,236}, and very few systems can effectively show this heterogeneity in such a high-throughput yet simple way (refer to 2.2.2).
Figure 6.16 Different activation patterns: single (a) and multiple (b) B3Z activators. Responders were gated from the same recording, where B3Z were exposed to K89 pulsed with 1 µM SL8.
Figure 6.17 Single B3Z responders. Cells were gated from the same recording of Figure 6.16, where B3Z were exposed to K89 pulsed with 1 μM SL8. An analysis was run amongst the single responders to see whether the first to activate would also be the cells carrying highest signals, but this assumption was not confirmed by the analysis, that proved a very mild correlation (top left). Top right: all traces overlapped, and their average reported in red. It is possible to see that the average signal is a poor representative of the single signal profiles (shown in the matrix below). This shows how important information can be lost when using techniques such as flow cytometry that report population averages. Bottom: individual traces of the single responders.
Figure 6.18 Multiple B3Z responders. Cells were gated from the same recording of Figure 6.16, where B3Z were exposed to K89 pulsed with 1 µM SL8. An analysis was run amongst the multiple responders to see whether the first to activate (first peak time) would also be the cells carrying highest signals, and a partial correlation was found (top left). Top right: all traces overlapped, and their average reported in red. Note how the average is not representative of the single signals, and these short and asynchronous activations could be easily overlooked during flow cytometry analysis. Bottom: individual traces of the single responders.

The next set of experiments was designed to measure the dose-response of T cell arrays at single-cell resolution. B3Z arrays were paired in the custom rig with K89, pulsed with 100 µM, 1µM and 10 nM SIINFEKL; and responses were recorded over 2 - 5 minutes. Data were obtained from different recordings, ran on different days. Activators were identified for each concentration and their traces were merged in three different matrices, plotted as heatmaps (Figure 6.19). While the magnitude of the responses to 100 µM SL8 appeared to be much greater than the other two sets, surprisingly only a small percentage of activators could be gated (9.77 %). Much higher percentages could be detected when cells were paired to 10 µM and 10 nM (respectively, 46.7 % and 24.4 %).

It was also noted that 92.63% of the responders that activated against 100 µM had single peaks, yet fewer (60.57%) of the responders that activated against 1 µM had single peaks (refer to Figure 6.16 and Figure 6.20 a, b for examples of both activation patterns).
profiles of T cells in this latter group generally showed multiple calcium peaks, each with a lower maximum than the typical single peak seen with higher abundance peptide stimulation. When arrays were exposed to APC pulsed with 10 nM peptide, the percentage of “single peak” activations dropped further to 46.88% (Figure 6.20 c, d report some examples of the two subgroups of responders). This is consistent with the fact that the B3Z cells are sensitive to peptide (i.e. reach a critical activation threshold) in the picomolar range (see above), and illustrates the increased depth of mechanistic detail can be captured using single cell array measurements: as peptide is titrated down, increasing numbers of cells are likely to achieve the activation threshold via “accumulation” of multiple sub-threshold spikes rather than a single major spike. Furthermore, as already noted, the low percentage of active-gated T cells stimulated with 100 µM peptide (close to 1000x the required for maximal stimulation in other assays – see section 3.2.1) may be a consequence of a pro-zone effect described in the literature, and recently modelled mathematically to describe bell-shaped T cell activation dose-response curves237.

While this platform could detect responding T cells efficiently and has throughputs that are 10-fold higher than that offered by existing platforms189 in terms of number of cells screened at once, it was noted that the system would benefit from improvement, as a lot of signals were confounded by noise arising from background interference during the recording. This might be improved in the future by either implementing more complex algorithms to track the maxima centres on each frame and follow small displacements of the cells, and also by enclosing the microscope in a dark room onto an anti-vibration mount, that could better stabilize the system. Inserting the platform in an incubation chamber, moreover, would be beneficial also for the cells responsiveness during the recordings, and responses could be monitored for longer periods. Lowering the background noise would also help in the activators detection, reducing false positives and speeding up the identification of subpopulations. On another note, adding a dye to control the APCs positions should be included in the standard protocol for future experiments, as the presented data were analysed under the assumption that being confluent, all wells should contain at least one K89 by the time of contact between the plates. An algorithm to match shared ROIs between the different channels could be easily implemented, as an extra precaution for the authenticity of the T cells activator percentages.
Figure 6.19 Time traces of single B3Z activators in response to K89 antigen presenting cells pulsed with different concentrations of SIINFEKL. a) activators stimulated with 100 µM peptide (results from 5 recordings, 9.77 % responders identified); b) activators stimulated with 1 µM epitope (results from 3 recordings, 46.7 % responders identified); c) activators stimulated with 10 nM SL8 (results from 3 recordings, 24.4 % responders identified).
Figure 6.20 Multiple and single responders. A selection of the traces from Figure 6.19 (a, c) is here pictured: multiple (a) and single (b) responders to K89 pulsed with 100 µM SL8; multiple (c) and single (d) responders to K89 pulsed with 10 nm SL8. Responses to K89 pulsed with 1 µM SL8 were instead reported in Figure 6.16.
On a final note, systematic recording of possible sources of artefacts during the recordings can be a very useful tool in downstream data analysis. An example is seen in Figure 6.10 and in Figure 6.12: when a fluorescence shift crosses all ROIs at the same time, it is clearly due to an issue with the acquisition system. For this reason, traces that showed a peak at such times were not considered as responders and excluded from the analysis.

A second example can arise from ROIs lined on the edges of the field of view. Due to the microscopic movements of the stage that can occur during the recordings, these cells can suddenly appear or disappear from the field, resulting in sudden fluorescent shifts that might be mistaken for activations. Part of this issue can be avoided by selecting “Remove Edge Maxima” in ImageJ when picking the cells. Nevertheless, these cells are quite easy to spot when all traces are plotted at once, and can be excluded from the analysis. While this artefact won’t cover all ROIs, it will most likely affect more than one, hence a few cells will be spiking and/or a few signal lowering within the same frame, and the following traces will be quite flat.

The last artefact was encountered during the first attempts to bring the plates together, and it is a quite useful feedback suggesting that the plates didn’t come together correctly. In fact, in a couple of recordings, no activations could be spotted a part from some sudden spikes in a few ROIs. Differently from the previous cases, these spikes wouldn’t happen at the same time, indicating the issue was not related to the time-lapse itself. This artefact arises from cells that didn’t set properly in the wells, hence when the plates are pushed together, they are shifted to the sides of the plate by the interstitial fluid, crossing the field of view. The sequential spikes are due to these “flying cells” temporary adding their fluorescence to the ones in the wells they are crossing. This event became clear by plotting the positions of the affected ROIs, when the trajectories of the flying cells became visible. An example is shown in Figure 6.21.
Figure 6.21 “Flying cells” artefact. This artefact happens when the two plates are not properly positioned, leaving a gap in between: cells that didn’t set properly in the wells will be pushed to the sides of the plates, crossing several ROIs in their paths. The detected signals will present sequential spikes (see graph of the single cells time traces). At the bottom, the positions of the affected ROIs were highlighted with yellow circles, revealing the trajectories of the “flying cells”. Scale bar 100 µm.

6.2.3 Discussion on heterogeneity

It has been mentioned in this study that three kinds of activation patterns could be highlighted: fast-burners, slow responders and multiple activators. While, due to the early stage of this research, it is not possible to explain the implications in the downstream signalling and potential different roles in the immune response, similar patterns have already been observed in literature. Multiple responses during T cell activation against second messenger signalling molecules such as H₂O₂ have also been investigated with a single cell resolution, showing high heterogeneity within clone lymphocytes populations. The group suggests the variability could originate from stochastic differences in gene expression, growth phase of the cells or epigenetic alterations. They also propose that maintaining a plethora of responses at a population level would be advantageous when mounting an immune response. When speaking about heterogeneity, it is important to make distinctions.
As thoroughly explained in 241, biological heterogeneity is a fundamental property of various cellular responses often masked by the use of a Gaussian distribution and average parameters to investigate the population. As mentioned in the review, heterogeneity could be genetic or non-genetic/phenotypic (as in the present study, that investigated clonal populations). Non-genetic heterogeneity can be divided into intrinsic or spatial, due to the micro-environment. The analysed setup aimed to provide a strictly controlled environment (isolated agarose wells, same agonist, synchronized stimulation) to avoid any spatial variation that could limit the comparability between readings. Anyhow, an analysis was run to investigate whether the variability in cellular responses could be a secondary effect to their positions on the plates (e.g. edge artefacts, or slow responders being activated by cytokines from neighbour responders), and it is reported in Figure 6.22. Fast responders (highlighted in blue) and slow burners (in red) were co-localized on the trapping arrays after antiCD3 stimulation (top image) or APC contact (bottom picture). In both cases no connection between the activators and the response pattern could be spotted. It is possible to identify slow responders both in proximity of fast burners or surrounded by non-activators. While the dark area to the right corner of the upper image might be related to improper pipetting of the stimulant, the two sub-populations co-exist in the left area of the array, suggesting that the difference is also not related to a mismatched stimulation time (e.g. due to slow diffusion). In the bottom figure, the homogeneous distribution of responders corroborates the thesis of a uniform micro-environment. Gough’s review 241 further distinguishes into micro-heterogeneity, where the investigated cell feature (calcium dynamics in this case) shows a broad spectrum of the same kind of response, or macro-heterogeneity, where discrete subpopulations can be highlighted. This classification becomes trivial in the distinction between fast burners and slow responders: while the distinct patterns can be more clearly spotted in Figure 6.12, in Figure 5.28 the variation appears to be less sharp, with some traces that could be identified as “in-betweeners”, and the definition of the two subgroups becomes more subjective when manually classifying the activators. In the case of multiple responders, as mentioned in 6.2.2, the correlation between percentage of multiple responders and peptide concentration suggests that in the presence of a low abundance of epitopes, cells are more likely to reach the activation threshold through multiple sub-threshold spikes. Lastly, a factor of influence that has not been investigated in this study and might be explored in future work is any possible correlation between calcium profiles and different phases of the cell-cycle.
Figure 6.22 Localization of fast burners and slow responders in the trapping arrays. After identifying the responders, and dividing them in two subgroups (fast burners, characterized by an early and sharp increase in intracellular calcium, here circled in blue, and slow burners, whose response was rising slowly to a lower plateau, here highlighted in red), the activators have been located onto the trapping arrays. a. Responders to antiCD3 and ionomycin stimuli, from Figure 5.28. b. Activators against APCs pulsed with peptide, from Figure 6.12.
6.2.4 Cell Patterning methods

One of the limitations of the current setup, is that the antigen presenting cells that are not facing wells arrays will be pushed against the agarose surface, resulting in cell stress or cell death, leading to the release of factors into the surrounding space that may confound accurate measurement of T cell activation. Moreover, it is possible that multiple APCs are displaced within the same well when they face the array. This could be avoided by patterning the cells onto the flat surface, with an arrangement that mirrors the well positions. This would of course reintroduce the need to align the plates before inducing the contact, with its attendant practical complications.

Nevertheless, I investigated cell patterning as a potential refinement of the technique. The first approach was undertaken in collaboration with Dr. Kian S. Kiang (Southampton Nanofabrication Centre, Faculty of Physical Sciences and Engineering, University of Southampton). The aim of the experiment was to test a polymer (PisC, from JSR Micro NV), whose hydrophobicity can be modified via UV-exposure. After the usual spin-coating of the adhesion enhancer TI, a 1 µm layer of the material was spin-coated onto a glass substrate; The uncured polymer was then exposed to UV light through an opaque mask containing transparent features, corresponding to the designs aimed for cell patterning. After post exposure baking (PEB), the exposed areas would recede creating micro-cavities characterized by a lower hydrophobicity compared to the rest of the surface. Wettability of exposed and unexposed areas was evaluated measuring the contact angle of the material: unexposed areas resulted to be hydrophobic (110°), while exposed areas were more hydrophilic (56°).

Two different adherent cell lines were tested for selective adhesion on PisC substrates, B16 and K89. The fabricated templates were placed into culturing wells, and each well was filled with 4 ml of media containing 1 million cells. The chips were then left in the incubator for cell culturing at 37°C with 5% CO₂ over a period of 48 hours.

Alternatively to the bare substrates, cells were also cultured on PisC-patterned slides coated with a mixture of Pluronic F-68 (P5556, Sigma-Aldrich) and fibronectin (33010018, Gibco). Pluronic F-68 (PEO-PPO-PEO triblock polymer) is a surfactant that prevents serum protein adsorption and consequent cell adhesion, and it is often used to coat PDMS surfaces in microfluidics for this property. Its hydrophilic PEO chains are in fact exposed to the culture media, while proteins tend to adsorb onto hydrophobic domains. Pluronic adsorbs better onto hydrophobic surfaces, so it should more likely bind to the unexposed areas of the PisC substrate. Fibronectin is an extracellular matrix protein that promotes cells adhesion binding to integrins at the cells surface. Widely studied in literature, it is known to have
higher adsorption onto hydrophobic surfaces, but an higher functionality once adsorbed onto hydrophilic substrates\textsuperscript{242}. Since literature suggests\textsuperscript{244} that PDMS surfaces must be plasma-treated to decrease their hydrophobicity in order to stamp fibronectin on them, and the elastomer has a similar contact angle to the unexposed PisC (100-107°), it was assumed that during fibronectin stamping the protein would rather attach to the exposed areas, or at least that it would have an increased effect when adsorbed over these features for the more favourable conformation for cell attachment that it would adopt on the hydrophilic substrate. Literature\textsuperscript{243} shows the difficult balance between pluronic and fibronectin concentrations to obtain the desired effect for cell attachment, and how the optimal formula can widely vary depending on the cell types. In these experiments, only one combination of concentrations was used (150 µg/ml fibronectin and 0.1% pluronic). The mix was added on top of the PisC substrate and 3 hours in a 37°C / 5% CO\textsubscript{2} were allowed for coadsorption of the molecules. Thereafter, the templates were rinsed twice using PBS in a wash bottle, and the cell solution was added as for the protocol used with bare substrates.

The best results were obtained on bare substrates for K89 cells and on pluronic/fibronectin coated surfaces in the case of B16, and are shown in Figure 6.23. Nevertheless, the reproducibility of these outcomes was low, and the chemistry involved in this patterning is complex due to the many factors involved: wettability of the surface and Vroman effect (competitive adsorption of molecules) will play a role in the initial coating of the substrate, influencing the actual ratio of fibronectin and pluronic exposed; serum proteins from the cells media can interfere with the cells attachment, as well as the cell type and specific adhesion preferences. Due to the complexity of this system, alternative patterning protocols were investigated. Also, although our cell line of choice (K89) could be patterned on the PisC substrate, they adopted an unusual morphology after the overnight incubation, suggesting that this polymer might not be the best choice for functional biocompatibility.
Figure 6.23 K89 (top images) and B16 (bottom images) patterned onto PisC featured substrates. For the top images, cells were deposited onto the bare cured polymer, while for the bottom images the polymer was incubated with Pluronic F-127 and Fibronectin, to enhance the likelihood of the cells to adhere to the patterned areas. Cells elongated more nicely on the fibronectin substrates, but while the pattern is clear in both cases, the reproducibility of these results was not optimal.

The second method of cell patterning was microcontact printing\textsuperscript{141,245,246}. This technique requires a stamp, generally made of PDMS, bearing the desired patterning. Polydimethyloxilane (PDMS) is a hydrophobic elastomer routinely used in microfluidics, and can be prepared by mixing two reagents (base and curing agent, typically at 10:1 w/w ratio), degassing the solution in a vacuum chamber and heat-curing the polymer mix on the relevant master. For these experiments, PDMS stamps were cured at 100°C for 1 hour on a hot plate, and off-the-shelf silicon masters from the Hybrid Biodevices Laboratory (University of Southampton, UK) were employed. A protocol from Cold Spring Harbor Laboratory Press\textsuperscript{141} was followed, and its main steps are schematised in Figure 6.24.
Figure 6.24 Microcontact printing protocol, adapted from\textsuperscript{141}. The PDMS stamp is covered with a drop of fibronectin solution and the protein is let to adsorb for 30 minutes (a); the protein excess is removed, and the stamp is carefully flipped onto a polystyrene oxygen-plasma treated Petri dish (b), where the pattern design will be transferred (c). A Pluronic F-68 solution is then added to the dish and let to adsorb onto the not-printed areas for 30 minutes (d), then the plate is washed with PBS (e). Eventually, the cell solution is added to the Petri, and after 2 hours of incubation unattached cells are removed from the dish. The plate is then put back in the incubator to allow cell growth (f).

At first, the patterning of a fluorescently-labelled protein (Tetramethylrhodamine isothiocyanate-conjugated Albumin from Bovine Serum (TRITC-BSA, A2289, Sigma Aldrich) was tested, to evaluate the feasibility of the protocol. An example of obtained pattern is shown in Figure 6.25. Hence, HeLa cells (a human cervical carcinoma cell line) were printed on the fibronectin/pluronic surface. The cell line was picked as it has also been employed in the reference protocol, showing good results. Figure 6.25 shows different patterns obtained with this cell line. High resolution of patterning could be obtained with this method (in the timeframe of observation, spanning between 12 and 36 hours), including features of single cell resolution. Given these results, microcontact printing seemed a promising method for the idea of patterning the antigen presenting cells in an arrangement that mirrors the wells of our device. Nevertheless, when the same protocol was repeated using K89 (and with multiple replicas), the outcomes were very different. The mouse fibroblast cell line did not sense the patterns but rather adhered to the entire surface. Also, although this technique has a great potential, the printing step (Figure 6.24, step c) is critical and requires many precautions, and would be a labour intensive procedure to add to the experiment preparation.
One last route was explored for cell printing, based on a technique called *stencil patterning*. This method relies on a mask having apertures that will correspond to the designs to pattern; the mask will adhere to the selected substrate, and fibronectin (or another protein for cell adhesion) is deposited on top. Thereafter, cells are added and left to adhere; once the pattern is covered, the mask can be lifted off. This technique has been used for patterned cell co-cultures (attaching a second cell type to the uncovered areas after peeling off the mask), to create cell aggregates that can be then retrieved or to guide cells differentiation, to cite some. Most of the published literature employs either PDMS stencils or cleanroom-fabricated membranes (e.g., in Parylene-C). To be noted that even when using PDMS stamps, either as a stencil mask or as a negative stamp to create one, cleanroom access is always required, increasing the overall cost and time involved in
the procedure. This is another limitation to the two alternative patterning methods introduced in this paragraph (selective surface modification and microcontact printing).

I developed a novel method based on the stencil patterning technique, which is economical and doesn’t require microfabrication skills or facilities, relying exclusively on a laser cutter and a plasma chamber.

The stencil masks were made of 100 µm thick membranes of Polyethylene terephthalate (PET). PET is a material commonly used in the production of cell culture inserts, with interesting properties for the cell stencilling: it has a low water absorption (contact angle 70°), that makes it adhere nicely to hydrophobic surfaces that need to be patterned (like PDMS or untreated Petri dishes) due to hydrophobic interactions; it can be sterilized using gamma radiation and it has good resistance to 70% ethanol; it is a stiff material, easy to handle. Experiments from the literature show cells growth both on untreated PET and laser-treated PET255. Laser micromachining is a widely used method in biotechnology, and allows to engrave several materials with micron resolution. The main advantage of using this method is the cost-effectiveness and rapidity of the design-to-device process, that produces a working device within few hours starting from scratch. It has also been used to create microwells on polyester films where to culture cells aggregates251, but never considered to fabricate reusable stencil masks for cells patterning to our knowledge.

Channels of different width and circular patterns with various radius were ablated onto PET sheets using the Epilog Laser Mini. The mask pattern was designed using CorelDRAW X6. Hairline setting was used to draw parallel lines interspaced 300 / 500 / 700 µm and 0.1 mm diameter circles with a 700 µm pitch. A useful method to guarantee a good cut without increasing excessively the power of the laser was to stack multiple lines/circles in the same position, so that the laser would pass several times on the same spot, leaving time to the material to cool down in between. As for the print settings, resolution and the frequency were kept to 1200 dpi and 5000 kHz, while power and speed of the laser were varied to find the best combination. Power ranged between 3% and 5%, and speed between 10% and 15%. Ablated features were observed down the microscope, and images were processed using ImageJ to calculate the average dimensions of the cuts. The average width of each line was obtained calculating the channel area and dividing this number for the X-size of the screen. Average diameters of the channels are reported in Table 6.1, and show that 4% laser power gives very variable channel widths, so better results can be obtained using 3% and repeating the cut a second time with 2% power if needed. More consistent results were obtained if 4-stacked lines were overlaid in the design of the mask.
Table 6.1 Ablated channels average widths depending on interspace between lines and laser power settings. “3+2” means two subsequent scans having nominal laser power of 3% and 2%. For 300 μm pitch, only the lowest power setting could be used without the membrane collapsing between the channels.

<table>
<thead>
<tr>
<th>Laser power (%)</th>
<th>300</th>
<th>500</th>
<th>700</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>87.25 ± 14.52</td>
<td>90.39 ± 5.58</td>
<td>77.12 ± 7.09</td>
</tr>
<tr>
<td>3 + 2</td>
<td>/</td>
<td>94.08 ± 4.08</td>
<td>85.03 ± 4.82</td>
</tr>
<tr>
<td>4</td>
<td>/</td>
<td>144.32 ± 15.10</td>
<td>147.99 ± 22.82</td>
</tr>
</tbody>
</table>

After sterilization, the plastic masks were adhered to untreated PDMS. These elastomeric flat substrates were obtained bonding a commercial PDMS membrane (0.05 mm UltraThin Silicone Film, Silex) to a microscope slide with 30 seconds of oxygen plasma (Femto plasma system, Diener electronic). Hence, the masked slides were exposed to oxygen plasma, to render them more hydrophilic, and coated with 20 μg/ml fibronectin for 30 minutes at room temperature (although cell adhesion was noted also in absence of the protein on the plasma activated surfaces). After rinsing the fibronectin excess with PBS, a suspension K89 in media containing 1% FCS was added to the substrate. Following a 2-hour incubation at 37°C / 5% CO₂ unattached cells were washed away using fresh media, the PET membranes were removed using tweezers and the slides were either covered with a coverslip for imaging or dipped in complete media and put back in the incubator to keep culturing. For the imaging, cells were stained using PKH26 membrane dye before being added to the substrate (as for protocol reported in 3.3). A schematic of the stencil method is reported in Figure 6.26.
Figure 6.26 Novel and cleanroom-free stencil patterning technique protocol. The PET membrane is ablated using a laser (a), then sterilized and adhered onto a PDMS substrate (b); PDMS surface is activated with plasma treatment, and fibronectin is added on top of the stencil mask (c); fibronectin excess is washed off (d) and cells are added to the substrates; after 2 hours, unattached cells are washed off (e) and the PET stencil is removed using tweezers; patterned cells are put back to incubate in growth media at 37°C / 5% CO₂.

Results of this patterning method are shown in Figure 6.27. While this method doesn’t allow to reach the same resolution as the previous ones, it proved to be the best patterning method for K89.

In summary, this paragraph highlighted some of the possibilities and limitations of various methods to pattern the selected antigen presenting cells, and resulted in a novel protocol to fabricate stencil masks whose benefits could extend to other research projects where 80 µm pattern resolution is sufficient, speeding up and lowering the costs involved in the process. While these experiments showed that K89 patterning would probably benefit from cleanroom-fabricated stencil masks, to reach the resolution needed to pair single cells to the lymphocytes trapped into the wells, they also proved that this method works better than others for cell lines that easily adapt and adhere to substrates, even if the conditions are suboptimal.
Figure 6.27 Stencil PET masks obtained with laser ablation and resulting patterns of K89. Lines of K89 were printed with 80 – 150 µm width and 300/500/700 µm pitch (a). Spots of K89 of 100 – 300 µm diameter were also printed (b), keeping the distance between dots at 700 µm. K89 were pre-stained with PKH26 membrane dye, and imaged with a fluorescent microscope.

6.3 Microfluidic membrane device

An alternative design to couple single arrayed lymphocytes with APCs was investigated.

The initial idea was to array single APCs in larger wells positioned on top of a thin elastomeric membrane. By pressurizing this membrane from underneath in a controlled manner, the deformation of the bottom of the wells would lift the cells, bringing them into contact with the T cells upon alignment of the two trapping devices.

The concept was inspired by the working principle of Quake valves256. Microfluidic devices containing these kind of valves are generally made of three layers of PDMS: a bottom layer, with engraved pressure channels; a thin membrane layer, effectively constituting the valves,
and a top layer, with the real microfluidic channels. The fluidic path can be controlled by opening or closing specific channels by pressurizing the correspondent valve channels in the bottom layer. When a pressure channel is activated, the membrane will be pushed and deformed, sealing the microfluidic channel in the top layer (see Figure 6.28 for a schematic of the working principle).

Figure 6.28 Quake valve working principle: cross-section and top view of the valve. When the valve channel is not pressurized, there is a flow in the microfluidic channel on top (a); when the valve is pressurized, the membrane deforms closing the channel above, and the flow is interrupted (b).

In this case, the Quake valve working principle was adapted as shown in Figure 6.29. The fabrication of such device involves the casting of two different PDMS layers, that can subsequently be plasma bonded: the bulk of the device, consisting of the pressure channels network, and the thin membrane that will host the cells, containing the well features. The fabrication protocol and limitations of this design follows.
6.3.1 Fabrication of the PDMS membrane layer

The prototype design saw the PDMS layer printed with well features, to host single APCs. Ideally, the membrane would be few microns thick at the bottom of the wells, that would add an extra 30 µm thickness, to help single cells sediment via size exclusion. Protocols to fabricate similar constructs can be found in the literature\textsuperscript{257}, as well as suggestions on how to handle large membranes without tearing them\textsuperscript{258}.

An SU8 master was used to cast the final structure. Afterwards, upon silanization of the master, the PDMS mixture was either cast or spin-coated on the patterned wafer; a weight placed on top of the uncured elastomer to guarantee a uniform thickness of the mold, and the membrane cured on a hot plate. Finally, the thin layer was peeled off from the wafer, and plasma bonded on top of the pressure layer, aligning the wells to the microfluidic channels. Tricks to align PDMS parts after plasma exposure can be found in literature\textsuperscript{259}, and rely on the wetting of the surfaces during alignment under a microscope, in order to maintain the temporary hydrophilic silicone oxide layer that allows the final bonding.

In order to support the weight used during PDMS curing at a uniform height, and also to protect the tiny pillars that will generate wells in the device, supporting structures were added throughout the array. These features were higher than the pillars by few microns, and this thickness characterized the deformability of the membrane on the bottom of the wells in the final device. In order to have features of different heights on the same wafer, two-step photolithography was required, as well as different masks for the two layers. The two layer design is shown in Figure 6.30. The bottom layer (a) of the silicon master contains the design...
that will be on the surface of the PDMS mold, while the top layer \((b)\) creates the SU8 pillars that will come into contact with the lid during the casting process. The pillar design is different in the bottom layer, since having supporting pillars in the master translates in larger wells in the PDMS stamp. These wells would trap most of the cells during the device loading. In order to prevent cell sedimentation by size exclusion, the pillars in the bottom layer were composed of several sub-structures of 10 \(\mu\)m width.

The master fabrication protocol was detailed in Chapter 3.5, and recipes 2 and 3 from Table 3.1 were used in sequence to fabricate the two layers, nominally of 27 \(\mu\)m (for the cell traps) and of 5 \(\mu\)m (arbitrary thickness chosen for the thin membrane at the bottom of the wells). The deposition of the second layer followed the post bake step of the first photolithography, and the SU8-5 photoresist was directly spun on top of the SU8-25 layer, without any TI prime deposition. Eventually, both layers were developed at the same time. An example of SU8 master obtained from the presented protocol is shown in Figure 6.30 (c).

Some critical steps of this protocol, and corresponding troubleshooting, were:

1. Spin-coating of the second layer of SU8. While the literature reports other parameters to consider for multi-layer photolithography\(^{260}\), what proved to be very influential was the combination of the two resists. In fact, when using SU8 from different series for the two layers (such as SU8-3025 and SU8-5), the different chemical formulation led to surface tensions during the second deposition, resulting in an uneven spread of the second layer. The problem was solved using resists from the same family (SU8-25 and SU8-5).

2. Alignment. While having a second layer of few microns (hence quite transparent) helped in spotting the alignment marks, what came to help was to use a marker on the bottom of the glass wafer to highlight their positions before the second spin-coating. Alternative tricks are to remove the SU8 on top of the marks either using tape during spin-coating or using a cotton-bud soaked in acetone afterwards.

3. Resist development. While the literature offers two options (either to develop the first layer before deposition of the second one, or to develop both at the end of the process)\(^{260}\), the unique and final development proved to work better for this procedure.
Figure 6.30 PDMS membrane design and fabrication. From the top: the mask to fabricate the two layers that will compose the membrane design (a, bottom layer; b, top layer); SU-8 structures on a silicon substrate. This master will be used to cast PDMS membranes, and the rectangles are intended to support the lid during the casting (c). PDMS device: the wells will contain single cells, while the supportive pillars are here exposed as small rectangles, with dimensions that should exclude cells sedimentation. Bottom row: some of the main limitations of this technique. It is trivial to obtain a 50 µm membrane both casting or spin-coating the elastomer (e, membrane obtained pouring PDMS onto the master, and peeling it off after solidification; f, membrane obtained by spin-coating a thin layer of PDMS onto a flat wafer). Also, such small SU-8 features are likely to tear during the peeling off of the membrane (g, an SU8 pillar, that detached from the master during the peeling of the PDMS membrane). One of the main limitations of this protocol is the handling of the wide but thin PDMS layer, that should then be aligned to the channels with micrometric precision. While all steps referred to literature\textsuperscript{257–259}, they are hardly implementable in a relatively fast and reliable way for disposable devices.

Once the master was obtained, casting of the thin PDMS membrane was attempted. Figure 6.30 (d) shows an example of elastomeric stamp obtained from the master. This step proved to be trivial in several respects. First of all, both spin-coating and casting of the PDMS were
attempted, nevertheless the resulting membrane always ranged between 80 – 120 µm while the aim was to reach less than 10 µm (refer to Figure 6.30, e and f). With this thickness, it is impossible to bend the membrane within the wells. Another major weakness of this design was the peeling off and handling of the micrometric sheet of PDMS. Before moving to the following steps to optimize, such as the alignment to the pressure channels and the surface functionalization necessary to help cell sedimentation into the wells, an alternative design of the device was evaluated.

By this point of the project, it had become clear that using APCs physiologically attached onto a flat surface rather than trapped in wells had several advantages. With results supporting this hypothesis (as for Paragraph 6.2.1), the fabrication of the microfluidic device was greatly simplified by having a flat membrane over the pressure channels rather than a multilayer structure with micrometric features. In fact, flat PDMS membranes of several thicknesses are commercially available, and sold as sheets adhered to rigid PET substrates, that can be easily cut to the right size and removed just after plasma-bonding to the bulk of the device. Hence, 20 µm and 50 µm Ultrathin Silicon Films (Elastosil) were purchased from Silex Ltd.

6.3.2 Fabrication of the PDMS pressure channel network

The purpose of the pressure channel network was to lift the APC monolayer uniformly in order to achieve synchronous and controlled contact with T cells across the entire array. With this in mind, a single inlet is required to control the entire network, since all channels should be pressurized at once. The chip was designed to cover an area of 2 cm², being the size of the agarose trapping plates. In this case, there were no requirements for the thickness of the PDMS stamp, as long as the surface was flat. In order to help the membrane to bend uniformly across the channels, and to enhance the chances that single APCs would come into contact with the trapped T cells, supportive pillars were inserted throughout the channels. These features should ideally anchor the membrane preventing its deformation in the interspace in between agarose wells. As each post in the microfluidic path adds a resistance causing a pressure drop along the line, and since the goal was to minimize the pressure difference to have a similar membrane deformation against all the wells, the channel design was modified to compensate for the resistance added by the pillars by enlarging the channel cross-section adjacent to the pillars. A Comsol simulation was run to demonstrate how the pressure drop is reduced with this design, compared to the straight channels. Results of these simulations are shown in Figure 6.31. In this simulation, the cross-section of a single channel was considered. It has to be noted that, while giving a good feedback on the design and geometry of the channels, this simulation is not fully representative of the actual behaviour as it doesn’t account for the membrane deformation. Due to the complexity of the
multiphysics and to the intrinsic experimental variability, I decided to quantify the actual membrane deformation based on experimental results. In the Comsol simulation to compare the two channels geometries, it was assumed a flow rate of 1 ml/min at the entrance of the device. Being 64 channels in parallel, it was considered that the actual flow rate at the entrance of the channel would be 15.6 µl/min. The entrance length of the channel (L) was obtained as L = 0.06*Re, where Re is the Reynolds number, that defines the flow in the channel. This number can be calculated as:

\[
Re = \frac{v \cdot Dh \cdot \rho}{\mu}
\]

Where \(v\) is the flow velocity, obtained from the volumetric flow rate (Q) and the area of the cross-section of the channel (A) as \(v = Q/A\); Dh is the hydraulic diameter of the pipe, and equals \(4*A/p\), \(p\) being the perimeter of the cross-section. \(\rho\) (density) and \(\mu\) (dynamic viscosity) are properties of the fluid in the channel. Assuming to fill the device with cell culture media, the density should be approximately 990 kg/m³, and the dynamic viscosity 0.00078 Pa·s¹. The two designs will have the same height (H), but different widths (W): 60 µm for the straight channel, 80 µm for the enlarged/shaped pipe.

Under these assumptions, the input values for the simulations will be:

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>50</td>
<td>4000</td>
<td>260</td>
<td>62</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>3000</td>
<td>220</td>
<td>55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Q [ml/min]</th>
<th>Q [m³/s]</th>
<th>V [m/s]</th>
<th>(\rho) [kg/m³]</th>
<th>(\mu) [Pa·s]</th>
<th>Re</th>
<th>L [m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0156</td>
<td>2.6E-10</td>
<td>6.5E-14</td>
<td>990</td>
<td>0.00078</td>
<td>5.1E-12</td>
<td>3.1E-13</td>
</tr>
<tr>
<td>0.0156</td>
<td>2.6E-10</td>
<td>8.7E-14</td>
<td>990</td>
<td>0.00078</td>
<td>6.0E-12</td>
<td>3.6E-13</td>
</tr>
</tbody>
</table>

Results are shown in Figure 6.31, and can be summarized as follows:

<table>
<thead>
<tr>
<th>Design</th>
<th>Pmax - Pmin</th>
<th>(Pmax – Pmin) / Pmax *100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straight</td>
<td>1782.87 Pa</td>
<td>1.73 %</td>
</tr>
<tr>
<td>Enlarged / Shaped</td>
<td>863.39 Pa</td>
<td>0.84 %</td>
</tr>
</tbody>
</table>
Figure 6.31 Comsol simulation to evaluate the pressure drop across a pressure channel. The 2D simulations aim to define the better design for the pressure channels network. The two images show the entrance of the channel (left, red colors) and the outlet (right, shades of blue). The legends indicate the pressure (Pa), and the simulations was run assuming a flow rate of 15.6 µl/min (corresponding to 1 ml/min at the entrance of the device, split into 64 channels) and a flow of culture media (ρ 990 kg/m³, μ 0.00078 Pa*s). Note that, due to the 2D nature of the simulation, the membrane deformation could not be considered.

Since the enlarged/shaped design generated the required reduced pressure drop along the channels, the mask was drawn using this outline. A detail from the final design is shown in Figure 6.32 (a).

For the master fabrication, a single step of photolithography was required. Recipe 4 from Table 3.1 was adopted.
Figure 6.32 Microfluidic device to bring APCs into contact with single T cells. Mask design of the device (a): inlet branching and magnification of the channels. Top view of a PDMS microfluidic device (b); the red dots represent the position that a 5*5 array of wells would assume when the modules are aligned. Cross-section of the microfluidic device (c), realized cutting a slice from a device with a scalpel.
PDMS was cast on the masters as usual: the two components were mixed at a 1:10 ratio, mixed and degassed in a vacuum chamber; the solution was then poured onto the printed wafers and cured for 1 hour at 100°C. The devices were then cut and peeled off from the master, and inspected at the microscope. Hence, the inlet (and outlet) of the device were punctured using a biopsy punch (ID 1.5 mm). A rectangle of the 50 µm thick Elastosil membrane was cut to cover the device, and one of the two protective sheets was removed. Casted device and membrane (with the exposed side facing up) were activated with O₂ plasma for 30 s and bonded together under a weight for 15 minutes at 80°C. An example of final device (top view and cross-section) can be observed in Figure 6.32 (b,c). Devices could be sterilized under UV light, and thoroughly wiped with ethanol 70%.

At this point, it is possible to remove the other protective layer from the PDMS membrane, that will require functionalization to guarantee cell adhesion. In fact, hydrophobicity of PDMS limits cell attachment and increases non-specific adsorption of biomolecules. In order to engineer the elastomeric substrate, a temporary hydrophilic silicone oxide layer can be generated using O₂ plasma. Fibronectin (150 µg/ml) was added to cover the area, and incubated for 30 minutes at room temperature. Hence, the fluidic tubing (1.6 mm OD, 0.1 mm ID, FEP, Dolomite Microfluidics) were connected to the punched holes, taking care not to pierce the membrane, and the device was ready for the experiments. Figure 6.33 shows the various fabrication steps of the PDMS device, and how it should work in combination with the agarose trapping plate. Growth of K89 on these devices gave great results, with a good adhesion and normal morphology of the cells, and an example is shown in Figure 6.34.
Figure 6.33 Schematic of the assembly and working principle of the pressure channels device. The PDMS membrane is plasma-bonded to the pressure-channels layer (a); the exposed PDMS surface is plasma-treated and treated glass coverslips are adhered to the sides of the device (b); the hydrophilic PDMS substrate is functionalized with fibronectin, cells are adhered and grown confluent onto the surface and the fluidic channels are plugged to the device (c); the device is ready to be paired to an agarose trapping plate containing the T cells (d). Upon alignment of the plates, the channels are filled with media and kept at resting state (no deformation of the membrane, e); once the channels are pressurized, they will deform pushing the adherent cells into contact with the T cells (f). Note that the minimum commercial coverslip thickness is nominally 100 µm, hence an alternative must be considered in case such membrane deformation cannot be achieved when pressurizing the device.
Figure 6.34 K89 grown onto a PDMS device. Cells were grown confluent onto the PDMS membrane, previously plasma activated and functionalized with fibronectin. The triangular shape of the cells suggests their healthy state. The yellow circles represent how the microwells array containing lymphocytes should be aligned to this device.

6.3.3 Measurement of the PDMS membrane displacement

In order to evaluate the membrane displacement, a system able to capture the Z-shift of the central sections of the channels was required. While it resulted very challenging to monitor this event with brightfield recordings, as it is hard to find focus on top of an undulated surface (as in the case of the device when the channels are pressurized), a great improvement in this analysis came from a paper found in literature, describing the work of Hardy and all\(^\text{261}\). The group studied the deformation of flexible PDMS microchannels under flow-induced pressure in a novel way: using fluorescence microscopy, they were flowing a fluorescent dye through the device, and using the linear relation between dye layer thickness and intensity readout they could calculate the actual deformation.

The relation that links the intensity of the emitted light (F) and the absorption path length (l) is described by Guilbault as \( F = \varphi I_0 (1-e^{-\varepsilon c l}) \), where \( \varphi \) is the quantum efficiency, \( I_0 \) is the incident radiant power, \( \varepsilon \) is the molar absorptivity and \( c \) is the molar concentration of the fluorescent dye. For low dye concentrations (\( \varepsilon c l < 0.05 \)), the relation becomes linear, and
can be expressed as $F = k \phi I_0 (1 + \epsilon_\text{cl})$. Literature suggests that the linearity approximation is correct for dye concentrations lower than 30 mg/l. Since $k$, $\phi$, $I_0$ and $\epsilon$ are constants and the dye concentration doesn’t change during the experiment, the brightness values will be linearly linked to the path length, and hence to the thickness of the layer of the flowing fluorescent solution. As a matter of fact, the light beam will cross “more sample”, and hence excite more fluorophores as the membrane is deformed.

The first experiments to evaluate this method were ran using Fluorescein dye (Sigma-Aldrich, F2456, MW 332.31) diluted in deionized water at a final concentration of 30 µM. It has to be noted that this dye photobleaches very rapidly, hence it was necessary to continuously exchange the liquid illuminated by the microscope to record any channels deformation using time series. In order to do so, PDMS devices were prepared, where both inlet and outlet were punctured and plugged to fluidic tubes. The inlet was connected to a syringe pump filled with the fluorescent solution, the outlet was left at atmospheric pressure, dripping into a waste tube. The device was mounted onto a PMMA laser-cut holder, that would keep it in position on the microscope stage. The device was tested flowing the solution at a constant flow rate of 1 ml/min, and the brightness change was recorded using time series, with intervals between frames of 2 seconds. Figure 6.35 shows the device connected to the tubing and clamped with a holder. Time series were analysed with ImageJ, using tools such as Surface Plot and Plot Profile; specifically, “Stack Profile Data” macro was downloaded to automatically save the intensity values of the selected cross-section over time as a csv file. Figure 6.36 represents an example of data being analysed.
Figure 6.35 PDMS microfluidic device connected to the fluidics and filled with food dye to enhance the contrast. The custom holder is made of PMMA. A similar structure, integrated with a base to fit the microscope stage, was used to keep the device still during the recordings, while the system was connected to a syringe pump, and a constant flow rate of 1 ml/min was set.

Figure 6.36 Intensity comparison when the system is at rest (a) and when pressurized at 1ml/min (b). The top images are raw frames from the same recording; the bottom graphs were obtained with ImageJ Tool Surface Plot, and help visualizing the brightness shift due to the membrane deformation when pressure is applied.
While opening an outlet helped in the dye exchange and hence to avoid photobleaching during the recordings, it also increased the pressure drop across the device. The membrane, in fact, will tend to bend more in positions closer to the inlet, while the pressure on the walls will drop substantially towards the end of the channels. This can be visualized in Figure 6.37, comparing the membrane deformation at the entrance of the device (a) and towards the end of the channels (b). During two subsequent recordings of the same device, the pump was started and paused in cycles, so that it could be possible to capture both resting state and the device under pressure. From the first graph, it is possible to notice how the membrane recovers the original shape when pressure is removed (comparing frame 6 and 66 of the recording), as well as how reproducible is the deformation, comparing two subsequent cycles of 1ml/min flow rate (frames 16 and 76). Nevertheless, a discrepancy between the first two and the last channel can be noticed. This is due to slight imperfections in the fabrication of the device (such as trapped dust, or some posts along the channel missing, or not completely bonded to the membrane). Moreover, as previously suggested, it is possible to spot how, under the same flow conditions, the channels will double their original height at the entrance of the device (30 µm shift), while the membrane will be deformed of 10 µm only on the other end of the device. This would obviously constitute a problem when facing this device to the agarose trapping plates, since ideally all cells should come into contact at the same time and under the same conditions.
Figure 6.37 Fluorescence intensity profile of 3 channels during the membrane deformation experiments using fluorescein. The dye was injected at 1 ml/min, and collected from the outlet in a waste tube. a) reports the fluorescence increase in proximity to the inlet. Channels are at rest in frame 6 of the time series; hence the membrane is pressurized (frame 16); the pump is then stopped, allowing the PDMS to recover (frame 66), and eventually started again (frame 76), showing a reproducible behaviour. b) represents the same analysis run on 3 channels close to the output of the system. The recording was started while the membrane was under pressure (frame 1), and the PDMS left to gain its original shape (frame 60). The actual deformations were worked out from the intensity values knowing that channels are 30 µm high.

A test was also run with an alternative dye, that wouldn’t degrade over time during the recordings, and hence could be used having only the inlet punctured in the device, and no openings for the outlet. In this case, a program was set to the syringe pump to infuse 100 µl at a rate of 20 µl/min. The same amount can be withdrawn following, thanks to the elasticity of the PDMS. To have a reference on the flow settings, the volume of the device was worked out from the mask design and the known height of the master. The area of each channel, obtained from DraftSight software, is 1.844 mm² (1844000 µm²); considering 128 channels for the entire device, it gives a total of 236.032 mm² (236032000 µm²). The inlet / outlet branching sections cover altogether 58.645 mm² (58644870 µm²); considering a device with 30 µm high channels, the total volume of the device can be approximated to 8840306100
µm³, that equals 8.84 µl. It has to be noted that infusing 100 µl in a chip designed to contain less than 10 µl should result in a membrane raise of 300 µm (ten times the height of the channels at rest). This, anyhow, is a wrong assumption due to the deformation of the channels also in the other walls. Although the deformation will be mainly in the z-axis due to the lower resistance, the bulk of the system is not uncompressible. Much more influential, moreover, is the major deformation that occurs in correspondence of the branching channels. Therefore, the measured deformations were lower than expected.

As a dye with stable fluorescence over time, blue food colouring was used (essential Waitrose Natural Blue Colour). Food colouring dyes are cheap and commonly used in microfluidics to test the chips, or for demonstration purposes. Blue food dyes are a solution of water, citric acid and spirulina. Spirulina is a cyanobacteria that produces C-Phycocyanin (C-PC), a protein that absorbs orange and red light (absorption peak 621 nm), and emits fluorescence with a peak at 642 nm. Although its spectral characteristics are known, the concentration in the product is not specified. Nevertheless, when tested, it showed linear behaviour (linear increase in fluorescence while linearly increasing amount of dye retained in the device, see Figure 6.38). According to these results, the membrane deformation between supportive pillars was around 7 µm after 300 seconds of inflow at a rate of 20 µl/min. This result highlights the necessity of inserting a rigid membrane in correspondence of the inlet and outlet branching channels, were, in absence of pillars that anchor the membrane, a much greater deformation was achieved.

The first attempts of pairing T cells in the agarose trapping device and APCs grown onto this fluidic membrane didn’t give reproducible results. This must be attributed to the already mentioned limitations of the system: it is hard to control such a wide area to have a flatness with micrometric precision; most importantly, since the two plates need to be kept at 7 µm distance from each other, there is the need to design a holding system that is extremely thin but rigid enough to support the entire chip. This task is almost impossible, but these results can be used for a new design of the chip, that should be greatly miniaturized to be more controllable. Anyhow, fabrication and functionalization protocols, together with the final characterization of the device have successfully been optimized, and can promptly be applied to the future generation of the chip.
Figure 6.38 Membrane deformation assessment using blue food colouring dye. This set of experiments was run filling the chip with dye at a flow rate of 20 µl/min and having no open outlet. The top figure represents the intensity profiles of 4 channels at various times in the test (processed with Plot Profile tool from ImageJ), and it is possible to see how initially the fluorescence doesn’t change, while it starts increasing after 200s (starting to deform the membrane). The middle figure collects the raw data of 8 channels recorded in different experiments, and shows how the behaviour is consistent. In the last graph, the same data are fitted using linear regression (with starting point restrained to zero), and show that the experiments were done in a dye concentration range where brightness and channel height could be linearly related.
6.4 Conclusions

In this Chapter, coupling single T cells with APCs is discussed.

In Paragraph 6.1, a method previously developed in our laboratory is examined. According to this system, single T cells and single APCs should be trapped in two agarose devices; hence, the plates would be faced and aligned, and the contact created lifting APCs loaded with superparamagnetic nanoparticles by creating a magnetic field gradient with a permanent magnet on top of the plates. While the nanoparticle loading proved to be efficient, limitations of the setup derived from the difficulty in aligning the two plates with the required resolution, and most of all from the inconsistent cells displacement from the wells.

Hence, in Paragraph 6.2 a new pairing mechanism is proposed. Being K89 an adherent cell line, APCs were grown confluent onto flat surfaces instead of being trapped into wells. This way, the cells-covered slide could be flipped on top of the agarose trapping plate containing the lymphocytes, creating cell-cell contacts in a much easier and more consistent way. The efficiency of the system was also increased, since APCs numbers were not limited by their wells occupancies. Several controls showed that the system was consistent, and lymphocytes wouldn’t activate in absence of the antigenic peptide (alone, against the bare substrates or against K89 not pulsed with the epitope). These results are shown both in 6.2.1 and in 6.2.2. In 6.2.2 it is also demonstrated that activators were effectively paired with antigen presenting cells, sharing the same wells. Numerous experiments showed that a population of activators could always be highlighted, also lowering the peptide concentration (the lowest tested concentration was 10 nM). The heterogeneity of the responses within the same clone population to the same cell line and antigen concentration was striking. Different peak values could be correlated with the time of peak of the cells, and different patterns of responses could be distinguished. Specifically, greater signals with a unique peak were more frequent in early activators and in presence of higher concentrations of peptide, while in presence of lower concentrations of antigen an increasing percentage of cells showed multiple responses with lower values.

A limitation of this setup comes from the fact that multiple APCs end up in proximity of the single lymphocytes, that is acceptable in the present study (a cell model with a single peptide), but would become an issue in experiments involving different epitopes. For this reason, different methods to pattern the antigen presenting cells have been attempted and are described in Paragraph 6.2.4. Modified substrate wettability, microcontact printing and stencil patterning were investigated. In the latest case, a novel method to pattern cells with a laser ablated stencil was purposed. This method proved to be the most efficient for K89 patterning, and would have the advantage of being cleanroom-free, making it accessible for
most biological laboratories. Nevertheless, the resolution limit that could be achieved with this protocol was not sufficient to pattern single cells. For this reason, while the choice of stencil patterning technique should be maintained, future work would involve the use of masks etched in cleanrooms for a single cell patterning resolution.

Another limitation of the suggested cells pairing method is that it requires a micromanipulator underneath the plates to regulate the relative positions of the devices; this makes the overall setup too bulky to fit most microscopes. For this reason, a microfluidic device was designed to evaluate the possibility to lift the APCs cells using pressurized channels. The fabrication protocol has been optimized and is reported in Paragraphs 6.3.1 and 6.3.2, and an innovative way to empirically quantify membrane displacement was successfully tested (as for data shown in 6.3.3). Drawbacks of this first generation of microfluidic device are discussed, and suggestion for a future device include scaling down the system and inserting a membrane that could work both as a spacer between the plates and to avoid channel deformation outside the designed areas.
Chapter 7: Conclusions and future work

This section will summarize the main results and achievements of the present study as a conclusion to the dissertation (Paragraph 7.1). In Paragraph 7.2, future directions to the work will be discussed, both in the case the goal will become single cells analysis on-chip or if the future findings will lead to the decision of retrieving specific lymphocytes from the platform.

7.1 Summary

This project addressed the need of biologists to have a platform that could allow to study and compare the signalling of thousands of individual lymphocytes in response to antigen presenting cells in a high-throughput manner. While flow cytometry is the gold standard method that can be used to evaluate time responses of cell populations, the technology lacks the possibility to follow the individual responses of single cells over time. Microfluidics and droplet technologies offer high-throughput methods to screen large numbers of T cell, but the design and customization of such devices is time consuming. The best microfluidic trapping device to our knowledge193 allows a time-controlled cell-cell contact, and a screening of hundreds of cell pairs in one go. The device fabrication requires multi-layer photolithography, and the design adaptation to different cell lines (different sizes) requires alterations of several dimensions of the traps. As for droplet microfluidics, while being a mature technology, it lacks the time control over the cell-cell contact, as the lymphocyte and APC are encapsulated within the same droplet, but the time to find each other is arbitrary; also, the actual number of correct cell pairs in droplets will be a fraction of the collected population, lowering the throughputs when the goal is to study and compare the first minutes of contact.

The platform introduced in the present dissertation offers an alternative way of bringing single lymphocytes into contact with antigen presenting cells. Specifically, thousands of single lymphocytes can be monitored over time, allowing identification of top activators and outliers for downstream analysis. The device is simple to design and adapt to different cell lines, since the diameter of the wells is the only size that requires modification, and the master fabrication is straight-forward, employing a single step of photolithography. Once the master is obtained, the entire study can be performed in any biology laboratory, as the devices are cast in agarose, and single cells can be trapped in the wells via passive sedimentation, without any need to use fluidics.

The thesis opens up with an introduction, in which project goals and platform requirements are discussed. Also, an explanatory section (Chapter 2: ) provides the reader with the key elements to understand lymphocytes activation mechanism as well as immunodominance
phenomenon. Specifically, the need for a device that allows high-throughput time recordings of single lymphocytes is remarked by the open questions on why certain antigens dominate the immune response. An in-depth overview of relevant literature on technologies that could be used to study this phenomenon follows.

Material and methods are summarized in Chapter 3: , while 3.5 develops the validation of the biological model used in the study. Specifically, dilution subcloning has been used to pick a population of B3Z (mouse lymphocytes) responsive to SINFEKL epitope. The T cell responses to soluble stimulants (ionomycin, antiCD3) were assessed using flow cytometry, and extracellular calcium influence was tested, showing the importance of running the experiments in growth medium, to provide an environment similar to the in-vivo conditions. Different antigen presenting cell lines were tested, and K89 proved to be the best to elicit B3Z responses, hence picked up for the model. It was proved that experiments should be run within two hours from the peptide addition, when the presentation on K89 surface is the highest. Interestingly, when testing the responses of B3Z to the peptide loaded APCs, it was proved that the force of the contact directly influenced the percentage of activators, demonstrating the complexity of factors that should be considered when investigating lymphocytes activation. Synchronization of the T cell responses is impossible to guarantee both co-incubating the cell lines in 96-well plates and using centrifugation, and subpopulations of responders cannot be distinguished using bulk population studies. In any case, these experiments proved the validity of the cell model, and that the first B3Z activations (calcium peaks) could be expected within 3 minutes from the cell-cell contact.

Chapter 5: introduced the agarose multiwell platform used to trap single lymphocytes. Design criteria and fabrication method were explained, together with the choice of the material. Cell viability in the devices was assessed, and the seeding protocol optimized to achieve the highest wells occupancies, higher than 90%, that will allow the recording of more than a thousand single lymphocytes over time in one field of view. The new data analysis protocol, based on standard and personalized plugins from ImageJ and a custom Matlab code, is then detailed. This post processing allows to identify the top responders choosing between different criteria, and to locate them on the plate. Experimental data is shown to prove the feasibility of the study using this setup, and T cell single activations to soluble reagents (ionomycin, antiCD3) are shown to be comparable to the ones observed using bulk analysis methods, but giving access to a deeper analysis of the data (actual percentage of responders, typical response profile, outliers).

Chapter 6: discusses possible setups that could allow the coupling of single T cells with APCs. The study successfully reinvented a first generation of a device previously developed in our laboratories, where T cells and APCs were trapped in agarose plates, that were then
faced to create the cell pairs. After proving few limitations of the initial setup, it was opted to pair the single trapped T cells with a flat surface where K89 were grown to confluence. Several controls showed that the new pairing system was consistent, recording T cells activations when paired to antigen-pulsed APCs, but no fluctuations in the calcium levels when the lymphocytes were pushed against the bare substrates or K89 not loaded with the epitope. The data highlighted heterogeneity of the responses even within the biological model employing a clonal population of T cells: lymphocytes activated according to three general patterns: either with a rapid single peak (especially when early activators, or in presence of higher concentrations of peptide); with multiple smaller spikes; or by slow accumulation to a (generally low) plateau.

While this setup fulfilled the main objectives, offering a way of pairing thousands of lymphocytes to peptide-loaded APCs, giving biologists a reliable yet simple platform to study immunodominance, some modifications were also suggested for future development, and partially investigated in the chapter. The first alteration would be to pattern the antigen presenting cells, to give the chance of investigating the response to several epitopes within the same field of view. Different patterning techniques were assessed, identifying in stencil patterning the most reliable method. A second alteration was considered if the use of micromanipulators underneath the devices would become a problem, and should a microfluidic chip be preferred. A microfluidic device was designed to test the possibility to lift the APCs adhered onto a thin membrane via pressurizing channels underneath. The fabrication protocol and an innovative way to test the actual membrane displacement are explained, and suggestion for a future design of the chip are included.

### 7.2 Future Work

In 6.2.4, the possibility to pattern single APCs have been addressed, and a potential method was selected based on preliminary experiments. Specifically, stencil patterning technique gave the most reliable results, although a cleanroom-resolution mask would be required. In 6.3, a first generation of microfluidic device that could help downscaling the setup to a size that could fit most microscopes was introduced. While the fabrication protocol and testing method of the device were defined and optimized, a new geometry to reduce the overall area of the chip, and a rigid yet thin spacer membrane should be embedded to move forward. As for the data acquisition, the introduction of an incubation chamber to the microscope would help under several aspects: it would be possible to record for longer periods, keeping the cells in a healthy state; the device would be more stable, eliminating background noise carried on in the signal processing. The data processing could be optimized afterwards,
where cleaner signals would help in the definition of algorithms automatically able to screen for certain profiles of responders. In this regard, APCs should be routinely stained with a Cell Tracker (compatible with Fluo8-AM, as minimum spill-overs could bias the selection of T cells); this would give a more accurate count of responders, as they would be gated amongst the cells pairs rather than over the entire lymphocyte population.

Nevertheless, all the mentioned suggestions aimed to optimize the cell-cell contact system. One major objective in my device design was to facilitate traceability and high content analysis of individual cells downstream of the activation measurement. Specifically, two main routes will be addressed: on-chip analysis (using Single Cell Western Blot technology) and single cell retrieval (via micromanipulator).

**7.2.1 Single Cell Western Blot**

Microwell arrays, such as the agarose trapping plates presented in this dissertation, have the ultimate advantage of having single cells embedded in a convenient conformation into hydrogels, that makes it feasible to integrate post-activation on-chip analysis (refer to 2.2.3). A very promising technology that became established in recent years is the Single Cell Western Blot (scWB), developed in Herr’s laboratory and documented in the literature^{92,191,263}. Briefly, an array of microwells is cast in photoactive polyacrylamide gel seated onto a glass microscope slide; after capturing single cells within the traps, chemical lysis is obtained with a denaturing cell-lysis buffer; polyacrylamide gel electrophoretic (PAGE) fractionation of the solublised proteins is then carried out and proteins are immobilized onto the gel using an UV-initiated process that relies on benzophenone methacrylamide co-monomer crosslinked to the polyacrylamide (PA) gel. Antibody probing can be achieved by diffusing fluorescently labelled antibody probes into the gel, that is eventually analysed using fluorescence microscopy.

I acquired hands-on training in this technique at the “Single Cell Analysis” course (Cold Spring Harbor Laboratory, June 2016). Although commercial platforms are available to carry out the entire process in an automatized way (Milo™, ProteinSimple) integration of scWB as a downstream analysis with our cell-cell contact system, would require customized tools.

To demonstrate proof of concept for in-situ single cell western blot I compared the expression levels of EGFP (Enhanced Green Fluorescent Protein, positive control protein) amongst single cells in a population of EGFP-transfected Neural Stem Cells (NSC), using β-Tubulin as a loading control protein. While we reported lower seeding in microwells when tested in our lab, the occupancies on the tested PA slide were high. The settling was achieved
incubating on ice a filtered solution of cells for 10 minutes. It is important to note that compared to a regular western blot, the concentrations of primary and fluorophore conjugated-secondary antibodies are much higher (1:10 of stock solution for the primary and 1:20 for the secondary antibody), nevertheless the required amounts are very small (30 µl each). Figure 7.1 shows the results after fluorescent imaging using a fluorescence microarray scanner. The picture was processed using the provided Matlab code. Similarly to the procedure described in this dissertation, ROIs were generated corresponding to each well, and the fluorescence intensity plots of the ROIs in the selected areas were averaged in a unique graph, where it was possible to manually select the peaks of interest and set a threshold to remove the noise. After that, resulting fluorescence intensity plots of each single well were generated, and manually selected good quality data was retained for further analysis. Protein expression was quantified by calculating Area-Under-Curve (Y:X), and possible correlation in the expression levels of GFP and tubulin among the population was investigated. No correlation was found in the tested sample.

Figure 7.1 Single Cell Western Blot. Fluorescence composite image of a scWB microwell array, obtained with a fluorescence microarray scanner. EGFP-expressing NSC cells were used; relative amounts of EGFP (green) and β-TUB (red) could be quantified upon fluorescent antibodies staining. Data processing can be achieved using custom Matlab scripts provided at CSHL (2016). Scale bar 100 µm.

When assessing the feasibility of Herr’s method in our laboratories, a mask was designed for the purpose. In fact, it would not be possible to use the same microwell array as the one employed in the cell-cell contact tests, since a larger interspace between the wells would be required to run the PAGE. As for the micrometric dimensions, the same distances reported
in 92 were adopted (a pitch of 500 µm in the direction of separation and 190 µm in the transverse direction). The microwells were organized in blocks of 4x9 arrays, and the size of each array was adapted to the field of view of a 4x objective mounted onto a fluorescence inverted microscope (Olympus IX81, 2160 x 1650 µm). Six blocks of these 4x9 arrays were organized in a 2x3 pattern to reach a total surface of 6400 x 6400 µm, being the dimensions of a well in a 96-well plate. Eight of these blocks were therefore aligned and spaced to fit a multichannel pipette loading of the samples. Five rows could be fit on a double-size microscope slide. This configuration potentially allows to test up to 8640 single cells at a time, that could be probed using 40 different antibodies (using multichannel pipettes and a 96-wells plate with the bottom removed on top of the gel to create the incubation chambers) and having up to 216 cells tested for each sample. The concept is schematized in Figure 7.2.

![Figure 7.2 Single Cell Western Blot mask design. Wells were organized in a 9x4 array that would fit the microscope field of view, while keeping the separation distances necessary to run the scWB. These arrays were grouped in 6 and patterned in 5 rows, to fit the loading of a multichannel pipette (8-tips) using a 96-well plate without the bottom positioned on top of a double-size microscope slide.](image)

A master was then fabricated using the usual photolithography. Recipe 5 from Table 3.1 was used. After the hard bake step, the master was silanized to make it more hydrophobic and prevent gel residues from sticking during the casting: perfluorodecylnitrilchlorosilane was added with 15 minutes of vapour deposition followed by 15 minutes of resting and 15 minutes at 80°C.

The PA gel needs to be casted on top of a microscope slide, in order to avoid loss of proteins due to diffusion underneath the wells both in the lysis and PAGE steps. In order to anchor the PA gel to the glass substrate, the glass microscope slides were preliminary treated using
a silane with methacrylate functional groups. The solution was prepared adding diluted acetic acid (1:10 in deionized water) to a 1:200 mixture of 3-(trimethoxysilyl)propyl methacrylate and ethanol with a 1:33 v/v ratio. Three minutes of incubation onto the glass slide at room temperature were sufficient to have a reliable coating. The slide was rinsed with ethanol, dried and ready for use.

As for the gel casting, a recipe containing 12%T (total acrylamide) was used. This percentage was selected under the assumption of an average molecular weight of the target proteins of 60 kDa. This fractionation range should be optimal for the measurement of three key proteins involved in T cell signalling (refer to Figure 2.3): ZAP70 (that gets phosphorylated straight after the TCR-APC contact), AKT (involved further down the cascade) and NF-kB (that detaches from a larger complex due to proteasomal degradation and moves to the nucleus to upregulate genes involved in T-cell development, maturation, and proliferation). Antibodies for these proteins both in their basal and activated states were available. While the final recipe to perform scWB should contain 3 mM BPMAC (the benzophenone compound required for protein immobilization, that can be purchased from PharmAgra Laboratories), the feasibility tests were run using a 12 %T, 2.7 %C solution of acrylamides, 0.1% Sodium Dodecyl Sulfate (SDS), 0.1% Ammonium Persulfate (APS), 0.04% Tetramethylethylenediamine (TEMED), 375 mM tris(hydroxymethyl)aminomethane (Tris buffer) at pH 8.8. Precursor mixture was sonicated and degassed in a vacuum chamber for 2 minutes before use, and it was pipetted into the gap between the glass slide and the master, allowing ∼30 s to wick through the gap. The gel was cured in a vacuum chamber filled with nitrogen, and following the slide was wetted on the edge using PBS and carefully levered using a razor blade.

When handling the gel, it was important to keep it hydrated, due to the high drying rate. Specifically, it was shown that within 7 minutes the wells would already deform noticeably (as observable in Figure 7.3). Cells loading and viability on PA slides was already discussed in 5.2.1: extensive washes of the gel are required to remove any toxic residual monomer to prevent rapid cells death. With due precautions, 80% viability was observed after 4 hours incubation on a flat gel. Well occupancies instead resulted to be much lower than the ones obtained with agarose.
Figure 7.3 Well deformation rate due to PA gel drying. A PA gel at time 0 (a) and after 15 minutes (b) incubating it at room temperature on the microscope stage. c. Graph showing the wells enlargement due to the drying over a time window of 25 minutes (mean and standard deviation).

7.3 Single cell retrieval

While microwell arrays offer the advantage of a possible integration of on-chip analysis, giving a potential unbeatable high-throughput screening over all traps within short time and limited hands-on, they also allow off-chip analysis following cell retrieval, that could pave the way to more in-depth data analysis of selected high responders. An interesting and relevant technology was brought to the market by Cell Microsystems, and hands-on training was offered at the Single Cell Analysis course at Cold Spring Harbor Laboratory in 2016. CellRaft System for Inverted Microscopes relies on a PDMS microwell device having magnetic polystyrene rafts at the bottom of each well. Single cells can be trapped in the wells (100 µm each side) using serial dilutions. The plate can then be moved to the inverted microscope, where it can be recorded in real time with a camera mounted on top. Below the plate, a needle is mounted onto the microscope light, and connected to a motorized system; positioning the stage in order to have the well of interest aligned to the needle, it is then possible to puncture the PDMS membrane and release the raft containing the cell; the same is then retrieved using a magnet, and released in a 96-well plate. Pictures from the well before and after release of the single cell were taken during the training, and are shown in Figure 7.4. This system is simple and high-throughput compared to alternative methods to retrieve cells from trapping devices; nevertheless, the fabrication would become more complex scaling down the wells, and the raft release would become much more difficult. In
addition, limiting cell seeding to serial dilutions significantly lowers the number of cells that can be screened at a time.

The traditional and alternative method that could be considered for future work on this project relies on the retrieval of single cells using a micromanipulator. An Eppendorf FemtoJet Programmable Microinjector (25 mm motorized range in X-Y-Z directions) was mounted onto an inverted microscope (integrated with an Extra-Long Working Distance condenser and a x4 phase objective) thanks to Dave Johnston (University of Southampton, Biomedical Imaging Unit) with the goal of testing the feasibility of cell retrieval from the agarose trapping plate. Pre-pulled glass capillaries (1 mm OD, 30 µm ID) were ordered from World Precision Instruments (TIP30TW1). It is clear from Figure 7.4 (c) that the array design would need modification to increase the wells interspace if working with capillaries of 1 mm OD (wells have a diameter of 20 µm and the current pitch is 15 µm). The other major problem is the access angle of the tip: while a vertical access would facilitate the suction of the cell from the well, in this position the arm would block the field of view. For this reason, the tip should also be bent; glass capillaries can be pulled and bent using a micropuller and a microforge\textsuperscript{264}. Although cell retrieval using micromanipulators is ultimately doable, highly-trained personnel would be required and the experimental throughput would be relatively low, making the protocol hard to implement and transfer between laboratories.

Although the intrinsic lower throughput compared to on-chip analysis, both technologies have huge potential for the study of single cells specifically selected amongst a pool, thanks to the most recent protocols that allow single cell genome amplification (see 2.2). Between CellRaft System and retrieval using a micromanipulator, the latter is more easily implementable with the cell-cell contact setup introduced in this dissertation.
Figure 7.4 Single cell retrieval. a, b. Single cell retrieval using CellRaft System for Inverted Microscopes (Cell Microsystems, Single Cell Analysis course, CSHL, 2016): selected cell before (a) and after (b) raft retrieval. The array device is made of PDMS, and magnetic polystyrene rafts are microfabricated on the bottom of the wells; when the cell of interest is selected, the PDMS membrane underneath the well is punctured to release the raft with the adherent cell; a magnet is used to retrieve the raft and release it in a 96-wells plate. c. Microwell array mounted onto the stage of an inverted microscope connected to a micromanipulator; 30 µm ID glass capillary on the left of the picture (out of focus). The setup was kindly customized by Dave Johnston (University of Southampton, Biomedical Imaging Unit).
7.4 Conclusions

This work introduced a new microwell array approach that enabled monitoring of time-controlled cell-cell interactions at the single cell level. The platform offers an innovative protocol to bring single lymphocytes into contact with antigen presenting cells, with higher throughput than alternative devices reported in literature.

Using the unique opportunity of monitoring thousands of single lymphocytes in a single field of view offered by microwell arrays, it enables a prompt identification of top activators and outliers based on their calcium signalling in response to various stimuli. More importantly, for the first time to the best of our knowledge, this device allowed to simultaneously pair the trapped T cells with APCs by capping the plate with a lid where the agonist cells were grown confluent. The simplicity of this mechanism, together with the adaptability of the trapping array to different cell lines (by optimization of wells diameter and depth) make this setup very promising, with great potential of application to other cell-cell interactions studies, that go beyond the present goal.

Another great advantage of the platform is that being an open system, it doesn’t require the bulky and expensive equipment usually necessary with microfluidics. This asset, together with the choice of agarose as a material to cast the devices, make the developed protocol very appealing for biology laboratories.

Starting from an earlier concept, that proposed to load the APCs with superparamagnetic nanoparticles to control the cell-cell contact magnetically, the fundamental improvements introduced by the new setup can be summarized as follows:

- The cell-cell contact can be simply and reliably induced by pushing the two plates (one containing T cells and the other loaded with APCs) together via a z-axis controller while at the microscope stage; there is no delay between the time of contact and the recordings;
- The cell pairs numbers were significantly increased, as they are not limited to the suboptimal wells occupancies of the APCs, due to the adherent nature of these cells;
- The cells positions are maintained either by the trapping wells or thanks to the APCs adherence to the appropriate surface, making the contact mechanism more reliable;
- New scripts for the data analysis were written, making the post processing faster (few minutes compared to a couple of hours) and more adaptable to large amounts of data;
- Scripts to identify the responders based on automatic or manual selection, to sort the signals depending on their characteristics (average, maximum within a time frame,
slope of the signal or specific thresholds) were developed, also giving the possibility to promptly trace the positions of the cells of interest within the plate.

A part from the novelty of the pairing mechanism, the microwell arrays hold great potential for integration with subsequent in-depth single cell studies. Being an open system, where cells are singularly isolated in wells, it offers a duplex future direction: lysis-on-chip would allow to perform single cell western blot on the trapped lymphocytes, while on the other hand it could be possible to retrieve live cells by manual picking of the relevant T cells using micromanipulators. This would pave the way to subsequent culture, or integration in any advanced biology protocol.

This project generated from the lack of technologies that allow to study immunodominance in T cells, limiting the understanding of the mechanisms that define the hierarchy of T cell activation. Gaining insight into the immunodominant subpopulation of T cells would help to modify and target the patient’s immune response.

The goal of building a platform able to trap single T cells and synchronously pair them with APCs, measuring the CTL activation over time was successfully met and, even using a biological cell model, heterogeneity of the responses within a clonal population of T cells could be highlighted. Three main patterns in the intracellular calcium signalling were distinguished: early activators generally displayed a unique sharp peak in the calcium level, slow activators responded with a delayed and lower plateau, and multiple smaller spikes could be highlighted especially when working with lower concentrations of peptide. These patterns could not be highlighted before using flow cytometry, that lacks the ability to follow single activators over time, and the throughput when using fluorescence microscopy was extremely low compared to studies that employ microfluidic tools.
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