Advanced cellular systems to study tuberculosis treatment

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Short title: Cellular models of TB

Abstract

Mycobacterium tuberculosis (Mtb) kills more humans than any other infection and drug resistant strains are progressively emerging. Whilst the successful development of new agents for multi-drug resistant Mtb represents a major step forward, this progress must be balanced against recent disappointments in treatment-shortening trials. Consequently, there is a pressing need to strengthen the pipeline of drugs to treat tuberculosis (TB) and develop innovative therapeutic regimes. Approaches that bridge diverse disciplines are likely to be required to provide systems that address the limitations of current experimental models. Mtb is an obligate human pathogen that has undergone extensive co-evolution, resulting in a complex interplay between the host and pathogen. This chronic interaction involves multiple micro-environments, which may underlie some of the challenges in developing new drugs. The authors propose that advanced cell culture models of TB are likely to be an important addition to the experimental armamentarium in developing new approaches to TB, and here we review recent progress in this area and discuss the principal challenges.

Highlights

- The need for novel approaches for studying tuberculosis is clear
- Several *in vitro* human granuloma systems have been developed
- These 3-dimensional models replicate different aspects of human tuberculosis
- Models can be used to study drug treatment and pharmacokinetics
- Lung organoid and chip systems have potential to deliver transformative approaches

Introduction

Tuberculosis (TB) is a major global pandemic, killing more people than any other infectious disease [1]. TB treatment is complicated by prolonged duration of treatment, ranging from six months for drug sensitive disease to 24 months for drug resistant disease, which is increasing in incidence. Therefore, it is widely accepted that a much stronger pipeline of new anti-tuberculous drugs is required. The current standard system of developing new antibiotics relies on the "3M's": Minimal inhibitory concentration (MIC), Mouse and Man [2]. This system has successfully identified new agents now clinically used for treating multidrug resistant TB, but the failure of recently studied treatment shortening regimes indicates limitations in this approach [3]. Each *in vivo* experimental model has benefits, but also limitations. For example, the mouse is widely used and has the benefit of being genetically tractable and relatively inexpensive, but the histology of wild type mouse granulomas differs from man and lacks hypoxia [4]. Novel models, such as the "Kramnik" C3HeB/FeJ mouse, develops hypoxic caseating granulomas, although with much higher mycobacterial loads than human granulomas [5]. The guinea pig and rabbit TB models are well characterised and form hypoxic granulomas, but are relatively limited by cost of housing and lack of immunological reagents [6]. The zebrafish model has the potential of high throughput [7], but uses *Mycobacterium marinum* as opposed to Mtb and zebrafish larvae lack T cells. The non-human primate model is limited by cost, throughput and ethical concerns [6,8]. Furthermore, it has been recently reported that extreme drug tolerance of *Mycobacterium tuberculosis* may occur in caseum [9], suggesting that it is important to use models that represent the diverse micro-environments encountered during human TB (Figure 1) [10].

Pyrazinamide is one of the most important front-line agents in the treatment of human TB, and was discovered relatively fortuitously. Significantly, it would have not have been discovered by current approaches used to develop new TB treatments [2]. Due to its structural similarity to nicotinamide, which showed some activity against mycobacteria in animal models, pyrazinamide was directly tested *in vivo* and found to be effective, bypassing nutrient rich selection where it would have been ineffective [11]. This indicates the need to develop and investigate novel systems that replicate the complex physiology of the host-pathogen interaction that occurs in human patients. Human granulomas are multicellular structures containing both inflammatory and stromal cells organised in 3-dimensions, with the matrix regulating the host-pathogen interaction [12] and often with central caseous necrosis. In addition, Mtb can be cultured from macroscopically normal lung tissue. Consequently, it seems likely that testing drug efficacy at the single cell level will not reflect the

complexity of microenvironments within humans. Considering the nature of clinical TB, we propose that the key attributes of such a system should include primary human cells, fully virulent *Mycobacterium tuberculosis*, multiple host cell types, three-dimensional organisation, prolonged duration of infection, high throughput and with the potential for dynamic environmental modelling and study of different micro-environments. In recent years, there has been significant progress in developing such model systems to study novel treatment approaches and we review these and then discuss future directions.

Emerging advanced cellular models

Formation of multicellular organised granulomas is a hallmark of TB infection. *Mycobacterium tuberculosis* is capable of residing within granulomas for a prolonged time asymptomatically during latent infection. Several researchers have developed *in vitro* 2-dimensional models of human mycobacterial granulomas [6,13]. Progressive recruitment of macrophages around live bacteria have been observed in these models, which reflects initial steps in host granulomatous response enabling cellular and molecular analysis of this event [14-16]. The complexity of interactions that take place inside human TB granulomas have been elegantly presented by Guirado and colleagues. They developed an *in vitro* granuloma model using human primary blood cells from individuals with and without latent TB infection and demonstrated that the granulomatous response was significantly different between the two groups [17]. Crouser subsequently demonstrated that mRNA expression patterns of granulomatous response from latent TB patients significantly differs from the molecular profiles of individuals with sarcoidosis [18].

Emerging concepts within TB granulomas are the importance of 3-dimensional (3-D) organisation and the regulatory role of the extracellular matrix [12,19], and 3-D models of *M. tuberculosis* granuloma have been developed. In these systems, infected primary human cells are co-cultured with collagen matrix gels, agarose beads or agarose-coated plates. Kapoor and colleagues created an *in vitro* model of human TB granuloma, which included infected peripheral blood mononuclear cells mixed with collagen and fibronectin [20]. In this system, Mtb dormancy was demonstrated, with subsequent Mtb resuscitation with immunosuppressive treatment. An alternative lung tissue model of TB has been generated by combining epithelial cells and fibroblasts embedded in collagen with Mtb-infected primary human monocyte-derived macrophages [21-24]. This system has been used to demonstrate that matrix metalloproteinase inhibition reduces granuloma size and bacterial load. To date, these *in vitro* granuloma models have not been used for high throughput drug efficacy testing, but have potential if further advanced.

In order to evaluate the efficacy of compounds intracellularly, high-throughput screening methods have been developed [25]. For example, the High-Content Screening Technology (HCS) has been utilised in the granuloma model developed by Altare, and demonstrated significant changes in the activities of compounds under extracellular compared to granuloma conditions [26]. In an alternative approach, Silva and colleagues developed a feedback system control (FSC) methodology based on a macrophage cell culture model of TB. This optimization platform was applied to identify improved drug-dose combinations for TB treatment [27], and led to identifying more efficacious regimes in the mouse model [28]. These impressive technological advances are currently based on 2-D cell culture systems that lack extracellular matrix, and one challenge is to move these approaches into 3-D.

Bioengineered microsphere model

Evidence is accumulating that eukaryotic cells cultured in 3-D are more representative of conditions *in vivo* [29,30]. We have developed a 3-D TB granuloma model based on bioelectrospray methodology and have used this to study efficacy of anti-TB drugs [31]. Microspheres are generated within a cell encapsulator, incorporating Mtb-infected primary human cells including monocytes and T cells within extracellular matrix (Figure 2). We have shown that granulomas form within the 3-dimensional matrix [32]. Significantly, pyrazinamide is bactericidal to *M. tuberculosis* in this microsphere system, but not in Middlebrook 7H9 broth or 2-D cell culture, demonstrating that antibiotic sensitivity within microspheres may reflect conditions in patients.

The microsphere system is highly tractable, permitting variation of cell content, extracellular matrix, sphere size, infectious dose and surrounding media [33]. One benefit of encapsulation within spheres is that it constrains cells and bacteria, preventing them being lost under flow conditions and thereby permitting pharmacokinetic and pharmacodynamic modelling of compounds using microfluidics and potential development as a high-throughput system. In the preliminary experiments, we combined the microsphere system with a prototype of a microfluidic plate and were able to model the effect of dynamic antibiotic concentrations on mycobacterial killing (Figure 3) [31]. In addition, this model can be applied to study emerging Host Directed Therapy (HDT) approaches for TB. For example, doxycycline, which is a licensed MMP inhibitor, suppressed extracellular matrix breakdown driven by Mtb [34]. As several thousand spheres can be generated from a single donor, multiple micro-environments can be studied. Furthermore, combination with dual encapsulation methodology would permit the modelling of the lipid-containing caseous granuloma centre within a collagen rich cellular capsule [35].

Hollow Fiber System

Another model in which the efficacy of anti-TB compounds have been extensively tested is the hollow fiber system. This model permits complex mathematical modelling and pharmacokinetic/pharmacodynamic studies mimicking human drug exposures, and the utility has been summarised by the developers [36]. These analyses inform calculation of bactericidal/sterilizing effect rates, and exposures associated with suppression of drug resistance can be identified using this system. To date, this model has been developed with Mtb in broth culture or with infected THP-1 cells or murine macrophage cell lines, without extracellular matrix. A recent development is the incorporation of the 3-D liver cultures embedded in alginate beads for hepatotoxicity assessment in babies, highlighting the potential of combining organoid systems with flow systems for *in vivo* modelling [37]. Further development is required to study drug penetration into multicellular lesions.

Lung organoid and chip systems

The complexity of human lung may be best represented with organoids as model systems. These human stem cell-derived 3-D structures comprise of native organ's multiple cell types and mimic the interactions occurring *in vivo*. Various studies have been carried out utilizing lung organoids to investigate human lung development and disease [38,39]. Organoids may contain diverse cell types, such as alveolar epithelial cells, thereby mimicking the complexity of cellular pharmacokinetic interactions *in vivo* [40]. Current systems tend to lack specific cell types such as macrophages, which makes them less suitable for study of infectious conditions at this point. Implementation of organoid technologies for high throughput screening during drug development may provide a more physiologically relevant platform [41-43]. Only recently, lung organoids have been investigated in the context of infection [44]. In that study, addition of the respiratory syncytial virus (RSV) to lung bud organoids (LBOs) recapitulated important features of human infection in this model. To date, we are not aware of lung organoids having been used to study Mtb infection, and significant further development is likely to be required to achieve this goal.

In recent years, organ-on-chip technologies have advanced rapidly and their potential for drug discovery has been highlighted [45,46]. These microengineered systems consist of microfluidic channels lined by living human cells. Similar to organoids, they are designed to mimic the functionality of living organs and therefore reflect the organised human-organ level pathophysiology

in vitro [47]. A technologically advanced system is the breathing lung-on-a-chip system created by Huh and colleagues [48,49]. In this model, human alveolar epithelial cells and pulmonary microvascular endothelial cells are co-cultured on opposite sides of a stretchable porous membrane to replicate the alveolar–capillary boundary of the breathing human lung. Furthermore, the tissue stretch that occurs during normal breathing is mimicked by the use of a vacuum. Upon infection with *Escherichia coli*, inflammatory responses of the human cells are observed, confirming that the organ-level functions can be restored in this system. This model has a great potential, but will need to be adapted to TB-specific conditions and optimisation for use in microbial containment.

In a parallel approach, Benam and colleagues have developed a small airway-on-a-chip, in which human lung inflammatory disorders such as asthma and COPD exacerbations can be modelled, along with evaluation of therapeutic responses [50]. This *in vitro* system consists of an upper layer of differentiated, mucociliary bronchial epithelium and a lower layer of microvascular endothelium to which fluid flow is applied. The authors replicated the COPD inflammatory phenotype on-chip by stimulating the epithelial cells with polynucleotide Poly I:C, which mimics viral double stranded RNA. This allowed testing of the efficacy of drugs and dissection of the mechanism of drug action at the molecular level in a human organ context *in vitro*. Therefore, both human lung organoids and chip systems have significant potential as models for studying TB infection and drug-efficacy. However, each will require sustained development to address the outstanding technical hurdles and provide granuloma models that mimic the multiple micro-environments that occur in human TB.

Conclusions and future directions

The recent disappointments in treatment shortening regimes indicate the need to build capacity in advance model systems to study TB to develop novel approaches. Human TB has multiple phases and microenvironments, and so a single model system is unlikely to address all requirements of drug discovery, such as combining high intricacy with high throughput. For example, a paucibacillary model with slowly dividing Mtb is likely to be required for studying latent TB, whereas a high bacillary load with caseating centre and hypoxic regions can be predicted to be required for cavitary pulmonary TB. An inherent tension exists between further development of complex model systems and the potential for high throughput or deployment in resource-poor high incidence TB settings. We feel that there have been exciting developments by combining primary cell culture modelling with engineering approaches. The efficacy of pyrazinamide in the three-dimensional bioelectrospray

system could be taken as a proof of principle that these models may be able to identify new agents that are active in the stress conditions encountered *in vivo* during TB. Therefore, such models may be able to deliver new agents, which may be key components of a true "short course" regime by targeting Mtb within a stressed environment. Potentially, advanced cell culture systems can be used to refine the number of candidate compounds at a relatively early stage in development, and also inform the most efficacious combinations. We envisage utility at the transition between initial *in vitro* development and commencing *in vivo* validation. This will have benefit both in terms of cost and reducing the number of animal experiments.

In terms of future developments, combination of advanced models with single cell sequencing may provide new insights into the host-pathogen interaction. Multi-parameter readouts can also predict efficacy of novel compounds both on the pathogen but also potential side-effects and host cell toxicity. Particularly for HDTs, multi-parameter readouts such as host cell survival, cytokine release and immunometabolism may be important, as each intervention may have diverse effects, some of which may be beneficial while others are harmful. The challenges to overcome are not only biological, since there are also specific engineering hurdles. For example, advanced fluid control manifolds are required to permit multidrug pharmacokinetic modelling in multiple wells over numerous days within biological containment laboratories. The authors propose that a central challenge is to identify which developments and innovations are the most critical to produce models that can predict events in patients, and this will ultimately determine how successfully compounds identified in model systems proceed to clinical trials.

Figure legends

Figure 1: The complexity of human TB granulomas. Mtb resides in different micro-environments with the granuloma, a multicellular structure organised in 3 dimensions with different extracellular matrix composition. Modelling antibiotic killing of Mtb *in vitro* may need to reflect all these micro-environments.

Figure 2: Primary human cells within microspheres. Mtb, red; monocytes, blue; T cells green. Reproduced with permission from [31].

Figure 3: Prototype microfluidic device for pharmacokinetic modelling around microspheres, with inlet and outlet channels.

References with special interest (•) or outstanding interest (••)

• Benam *et al.* 2016 [50]:

Development of a small airway-on-a-chip, allowing for modelling of human lung inflammatory disorders and testing the efficacy of drugs at the molecular level in a human organ context *in vitro*.

•• Bielecka *et al.* 2017 [31]:

This study showed that Mtb is pyrazinamide sensitive in the microsphere system and that pharmacokinetic modelling around microspheres can be performed.

• Chen *et al.* 2017 [44]:

In this study, key features of lung development are established using the lung organoid model created from human pluripotent stem cells and human viral lung infection is investigated.

• Crouser *et al.* 2017 [18]:

Development of an *in vitro* granuloma model for studying disease mechanisms and treatment in the context of TB and sarcoidosis.

•• Guirado *et al.* 2015 [17]:

Investigation of PBMCs from patients with latent and active TB in a granuloma model, analysing both host and microbial outcomes.

• Huh *et al.* 2015 [49]:

Development of “breathing lung-on-a-chip” device, which reproduces the functional unit of the human living lung with the potential to model complex human disease processes.

•• Kapoor *et al.* 2013 [20]:

Development of an *in vitro* human tuberculosis granuloma model for studying dormancy and resuscitation mimicking features of the human disease.

•• Parasa *et al.* 2017 [22]:

Investigation of a multicellular 3-D model incorporating macrophages, epithelial cells and fibroblasts, with investigation of the functional effect of matrix metalloproteinase inhibition.

•• Silva *et al.* 2016 [27]:

Development a high-throughput macrophage model and its application to improve regimens for treatment of tuberculosis.

•• Silva-Miranda et al. 2015 [26]:

Application of high-content screening technology merged with a human granuloma model for investigating of the activities of anti-TB compounds.

•• Srivastava *et al.* 2016 [37]:

Combination of the hollow fiber system with a liver organoid to investigate treatment of childhood TB and toxicity.

• Tezera *et al.* 2017 [32]:

The original description of the characteristics of the bioelectrospray model and investigation of the host-pathogen interaction.

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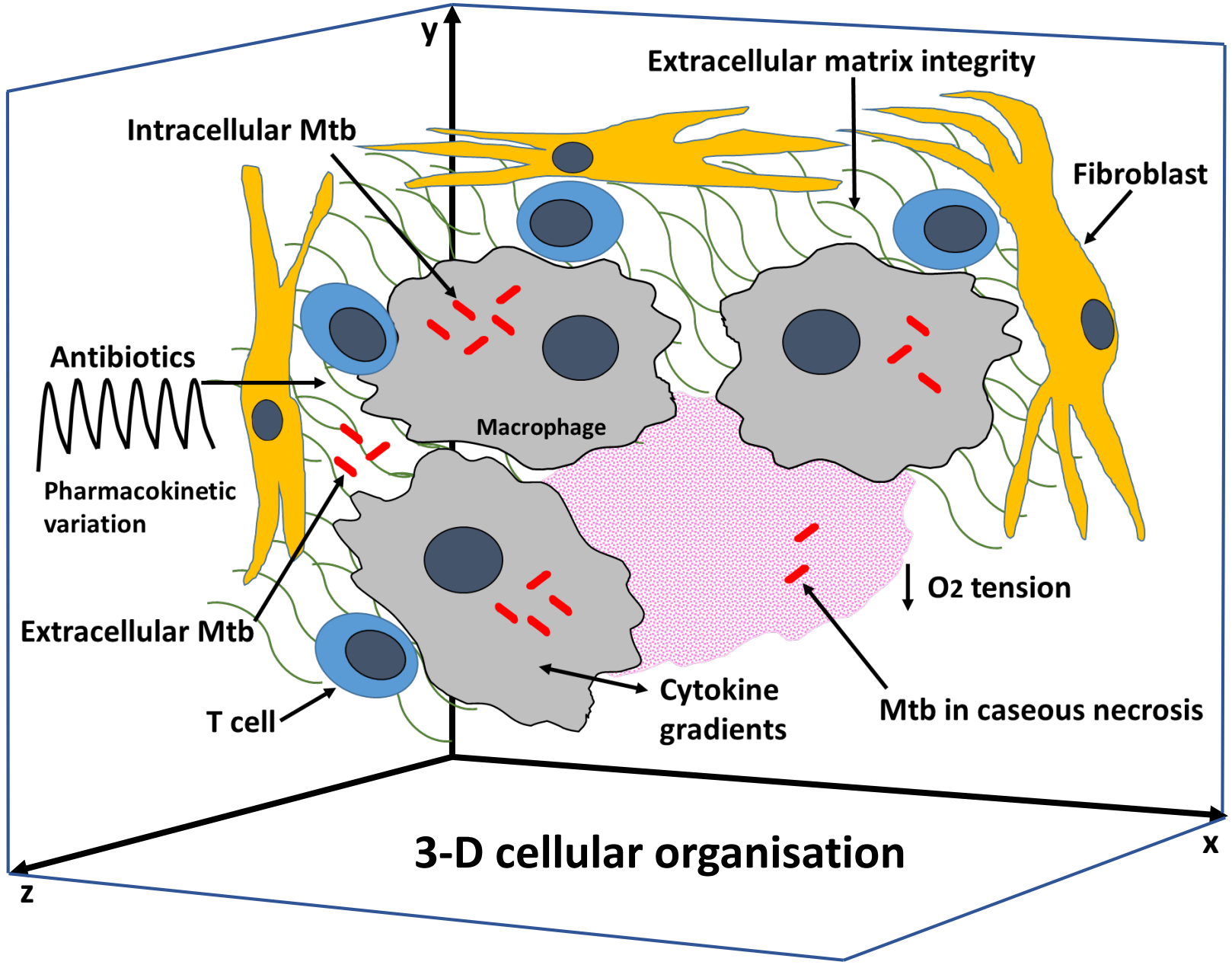
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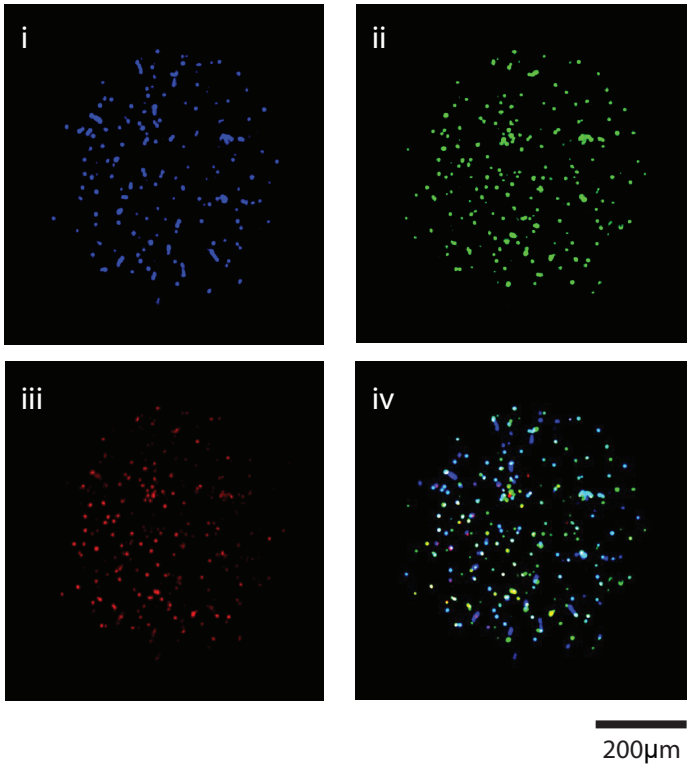
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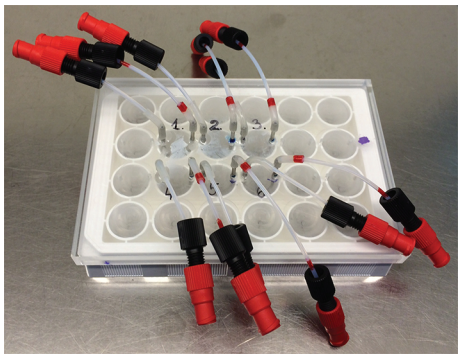
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