*ATVB/2021/317092 revised 03/03/2022*

**Emerging Technologies for Understanding Platelet Diversity**

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**Abstract**

This review discusses our understanding of platelet diversity with implications for the roles of platelets in haemostasis and thrombosis, and identifies advanced technologies set to provide new insights. We use the term diversity to capture intra-subject platelet variability that can be intrinsic or governed by the environment, and lead to a heterogeneous response pattern of aggregation, clot promotion and external communication. Using choice examples, we discuss how the use of advanced technologies can provide new insights into the underlying causes of platelet molecular, structural and functional diversity. As sources of diversity, we discuss the proliferating megakaryocytes with different allele specific expression patterns, the asymmetric formation of proplatelets, changes in platelets induced by ageing and priming, inter-platelet heterogeneity in thrombus organisation and stability, and platelet-dependent communications. We provide indications how current knowledge gaps can be addressed using promising technologies such as next-generation sequencing, proteomic approaches, advanced imaging techniques, multi-colour flow and mass cytometry, multi-functional microfluidics assays, and organ-on-a-chip platforms. We then argue how this technology base can aid in characterising platelet populations and in identifying platelet biomarkers relevant for the treatment of cardiovascular disease.

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| **Nonstandard Abbreviations and Acronyms** |
| **NGS** next-generation sequencing |
| **MK** megakaryocyte |
| **iPSC** induced pluripotent stem cell |
| **scRNA-seq** single-cell RNA-sequencing |
| **CITE-seq** cellular indexing of transcriptomes and epitopes by sequencing |
| **STORM** stochastic optical reconstruction microscopy |
| **DBiT-seq** deterministic barcoding in tissue for spatial omics sequencing |

**Introduction**

Circulating platelets continually monitor the integrity of the vessel wall and instantly respond when haemostatic or pathological vascular conditions dictate them to do so. There is an emerging recognition that platelets differ in terms of ultrastructure, protein composition, reactivity and functionality1, lending weight to the concept of different platelet populations with distinct roles in health and in disease. However, despite the cumulating datasets, we have yet to determine the nature of a 'hyper-active' or ‘pathogenic’ platelet. We reason that, to address this challenge, new technologies are needed to define better disease prediction and treatment. **Box 1** provides a brief description of the advanced technologies covered in this review. Below, we will focus on multi-colour flow and mass cytometry, next-generation sequencing (NGS) read-outs, proteomics, advanced imaging techniques, microfluidics and new organ-on-a-chip platforms.

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| **Box 1: Brief description of advanced technologies** | |
| ***Multi-Colour Cytometry*** | Spectral multiplexing using antibodies to label molecular targets on and within cells with data acquisition using flow, image and mass cytometry approaches. |
| ***Next-Generation Sequencing*** | A tool-kit involving sequencing read-outs capturing genomic, transcriptomic, phenotypic and also spatial information, each enabling single cell characterisations. |
| ***Organ-on-a-Chip Models*** | Engineered cell-based systems which recapitulate *in vivo* conditions, ranging from whole organs (*e.g.* bone) to discrete physiological components notably hydrodynamic shear and platelet activation sites for the purposes of this review. |
| ***Advanced Imaging*** | An umbrella term encompassing super-resolution imaging, multiphoton excitation and non-linear approaches capturing ultrastructural and biochemical information. |
| ***Proteomics*** | Proteome profiling using mass spectrometry approaches applied to bulk cell samples, with new innovations including nanopore sequencing having the future potential for single- of few-cell proteome measurements. |
| ***Microfluidics*** | Fluid manipulations in microchannels for analytical applications, including cell sorting, sample focussing, assay multiplexing and single cell confinement. |

The review is structured around prominent questions related to the different sources of diversity throughout the lifespan of circulating platelets (**Figure 1**). The following sections describe: (1) the various origins of platelet diversity, (2) how platelet ageing and priming impacts diversity, and (3) the spatiotemporal diversity of platelets in thrombus formation. For each section, we provide an update of our current understanding based on established assays, and we discuss how multiple advanced technologies promise to provide deeper insights into the differences in molecular composition, structure and functionality between platelets and platelet populations. As background, most findings are from human or mouse platelet studies. In the final section, we describe how new, technology-driven discoveries can identify platelet biomarkers and populations contributing to cardiovascular disease, and aid in disease treatment.

**1. Origins of Platelet Structural and Functional Diversity**

Here we explore the contributions of megakaryocyte (MK) heterogeneity, the asymmetry in pro-platelet shedding and subsequent platelet formation to structural and functional diversity with examples of clinical relevance.

***1.1. Proliferating megakaryocytes as a source of platelet diversity***

Platelets are derived from MKs, with recent research pointing to substantial heterogeneity within MKs in the bone marrow of a given subject, providing an important source of platelet diversity. The contribution of platelets reported to be derived from MKs in the mouse lungs2, is likely to be small but remains unclear. The evidence for diversity of circulating platelets comes from human and murine single MK transcriptomic analyses3 and functional assays4,5. For instance, in myelofibrosis, transcriptome profiling and mass cytometry examinations identified a MK progenitor sub-fraction comprising distinct mutant clones6. Overall, this heterogeneity raises the question to what extent the MK make-up can dictate platelet diversity in terms of structure and function7.

There are some experimental challenges for answering this question. A first challenge is to obtain in sufficient numbers a pure population of the rare (0.05%), large (~50 m) and fragile MK cells. This requires fluorescence cell sorting with large (200 m) nozzles or delicate magnetic cell sorting. The large MK size also prompts the possibility of using inertial microfluidics for high throughput size-dependent cell sorting8,9, potentially allowing the isolation of several hundred purified MKs per minute. Alternatively, isolated MKs or induced pluripotent stem cells (iPSCs) can be cultured in scaffolds or in bone-on-a-chip models to provide an artificial haematopoietic-like niche10. However, even the use of single-cell-derived iPSC clones requires stringent control to manage differentiation drifting11. More work is needed to demonstrate that such well-defined MK populations and their shed proplatelets provide an authentic recapitulation of their *in vivo* heterogeneous characteristics.

In addition to intrinsic MK properties, acquired and environment-induced features need to be taken into consideration. Single-cell RNA-sequencing (scRNA-seq) was used to demonstrate age-associated alterations in MK populations in mice, highlighting a versatile reprogramming of inflammatory, metabolic and mitochondrial expression pathways12. These MK features could be linked to given platelet phenotypes, for instance indicating hyper-reactivity to thrombin. By extrapolation, this suggests that localised MK perturbations in the bone marrow can give rise to platelet heterogeneity. New experimental concepts, for instance employing bone-on-a-chip models10 and using growth factor and other environmental-altered MK culture conditions will result in (pro)platelets with likely functional heterogeneity. The bone- and other organ-on-a-chip technologies relevant to platelet biology are described in **Figure 2** and compared with the microfluidic analytical methods suited for platelet research.

Megakaryocyte effects on platelet diversity can also be understood by lineage tracing via genetic-barcoding of murine MKs using, for instance, CRISPR-Cas9 reporters13. Such techniques will provide NGS read-outs for linking platelets to their parental cells. It then becomes possible to classify platelet populations from differences in the transcriptomes, and link the populations to the outcome of functional assays. This approach will answer the question whether dissimilar platelets are originating from a common or distinct origin.

***1.2. Allele-specific and clonal expression patterns affecting platelet diversity***

Efforts to catalogue human genetic variants such as the 1000 Genomes Project14 and the Simons Genome Diversity Project15 have identified up to 4 x 107 heterozygous single nucleotide or other variants, with a considerable fraction leading to deduced mRNA and protein variation. Human MKs and platelets contain an estimated 14.8k protein-encoding gene transcripts, producing an achievable platelet proteome of approximately 10k proteins16. Accordingly, with so many frequently occurring single nucleotide variations (SNVs) together with a large set of potentially pathological mutations, heterozygosity can be expected in almost every mRNA and protein sequence in the healthy human population. Indeed, public protein databases (UniProtKB, GeneCards, Ensembl) and human disease databases (OMIM) show common sequence differences in the majority of intracellular and secretory proteins. However, surprisingly little is known on how this 'universal' heterozygosity extends to inter-platelet variations within a given subject. Conventional cell biology considers that per cell only one allele is expressed, but whether all MKs, which are polyploidy, preferentially express one or another allele is unclear. For disease-linked SNVs and mutations this is an especially important question to answer, with new technologies poised to resolve this in several ways.

Recent developments in scRNA-seq have indicated that 12–24% of the expressed genes are mono-allelically expressed during mouse development, while about 75% of the heterozygous loci express only one allele in single primary human fibroblasts17,18. This issue is complicated by time-dependent transcriptional bursting, which is assumed to be a major source of variability in single cells19. Accordingly, in platelets, even derived from the same MK precursor cell, transcripts and proteins from one or two alleles may be present per cell. How this leads to heterogeneity in mRNA and protein expression in individual platelets and in the whole platelet population is virtually unknown. From the current literature on human platelets we can only deduce that considerable inter-subject differences can exist, possibly due to overall differences in allele-specific transcription. For instance, in the autosomal recessive disorder of Glanzmann thrombasthenia, in which compound heterozygosity of mutations in *ITGA2B* and/or *ITGB3* lead to a bleeding phenotype, flow cytometry has demonstrated different abundance levels of integrin IIb3 per platelet20.

In dominant gain-of-function mutations in the *STIM1* an *ORAI1* genes, which give rise to a combined immune and platelet disorder, the same heterozygous mutation even within one family can result in markedly different platelet phenotypes21,22. This implicates the presence of inter-subject differences, likely combined with intra-subject differences in allele-specific gene transcription. Along the same line, the platelets from individual patients with a heterozygous mutation in the *GNAS* locus, controlling the Gs protein and thereby adenylate cyclase and protein kinase A (PKA) activity, show clear differences in PKA-dependent phospho-proteome patterns, which accompany altered platelet properties23. A distinct example concerns a regularly observed somatic JAK2 mutation V617F in patients with polycythaemia vera associated with thrombocytosis and clonal haematopoiesis24. To what extent the qualitative and quantitative changes in platelets in these patients depend on the degree of expansion of mutated MKs and the derived platelets is still unknown. Based on these and many other examples, we envision that detailed knowledge of the penetrance of SNVs or mutations in the mRNA and protein profile of circulating platelets will fundamentally increase our insight into genotype-phenotype relationships of platelet-related disorders.

Several forefront technologies have the potential to deliver such new knowledge: *(i)* RNA-seq can be performed on platelets from a given subject, in particular by comparing expression of the mutated mRNA with a core of stably expressed mRNA species. The emergence of CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing)25, in which proteins are detected using oligo-labelled antibodies and NGS allows allele expression to be linked with protein levels at single-cell resolution. *(ii)* Multi-colour flow cytometry can be used with probes which specifically recognize the mutated or variable protein form. *(iii)* Targeted mass spectrometry allows stable-isotope labels to be spiked in for each mutated or varied protein form. We foresee that a combination of molecular characterisation methods applied to single platelets will reveal to what extent functional diversity can arise from variable allelic expression.

***1.3. Asymmetric platelet formation causing ultrastructural diversity***

In mouse, it has been shown that MKs shed proplatelets by an elongation process, which is driven by microtubule cytoskeleton rearrangements26. During proplatelet shedding in mouse and the maturation of barbell-shaped proplatelets in man, the final platelet size is considered to be the result of hydrodynamic shear stress on microtubule bundling, elastic bending and actin-myosin-spectrin cortex forces27,28. As this shedding process is highly asymmetric, it produces human platelets with widely different sizes, on average 2–3 m in diameter, with a volume coefficient of variation of >60%29. In conventional flow cytometry single platelet size estimates are obtained from Mie's theory of light scattering profiles, and can correlate with phenotypic variations30. As recently reviewed31, larger human platelets with higher density obtained by differential centrifugation, may have higher glucose metabolism and lipid peroxidation, have more ribosomes, ATP-containing dense granules and membrane glycoproteins, and often show stronger activation characteristics. Although not universally accepted, a high mean platelet volume has been associated with risk of cardiovascular disease32,33. This may or may not be due to altered proplatelet shedding leading to differences in the abundance of organelles. In light of this, it is important to more extensively study the relation between size and functionality of single human platelets, in terms of adhesion, spreading, secretion and aggregation. Such insights can be obtained by cell size markers, multi-colour live imaging studies, such as flow-cam imaging34, vessel-on-a-chip methods35 and protein micropattern printing36. All of these approaches can generate information at substantial throughput, allowing a comprehensive description of the size-function relationship for the characterisation of large platelet populations.

It is assumed but not proven that platelets are similarly packaged with abundant soluble proteins and RNA species, whereas the amounts of rare molecules and organelles like granules, rough endoplasmic reticulum, mitochondria and lysosomes are subjected to stochastic distribution. Based on the small platelet volume and considerable size heterogeneity, this can result in marked platelet diversity with functional consequences. For instance, an enrichment in mitochondria can impact platelet lifespan and survival under conditions of stress37. So far, ultrastructure organelle measurements by scanning electron microscopy (SEM) have provided some insights into the expected diversity of human platelets. In a recent study involving SEM, organelle enrichment in young platelets was correlated with higher transcript levels and higher reactivity to agonists38. A drawback is that electron microscopy requires sample fixation, thus removing dynamic information, and is time-consuming, thus limiting the ability to broadly survey platelet populations and characterise diversity. On the one hand, classical multi-colour fluorescence microscopy can be used to obtain high throughput structure-function information39, but it cannot resolve platelet organelles and large molecular assemblies. This requires optical resolution beyond the diffraction limit.

In the last decade, super-resolution microscopy methods, incorporating various advanced optical principles, have greatly improved resolution and throughput, while also becoming affordable. Structured illumination microscopy (SIM) with 120 nm resolution has been used to image organelles and platelet granules of fixed, fluorescent-stained platelets, and applied to the diagnosis of Hermansky-Pudlak syndrome40,41. Emerging computational and deconvolution approaches improve the resolution to 50 nm, and by combination with expansion microscopy approaches the resolution is further pushed to 20 nm42. High-resolution imaging of live platelets is feasible using stochastic optical reconstruction microscopy (STORM), which even visualises the morphology and organelle dynamics upon platelet activation43. Discussed in more detail in section 3.2, STORM opens the possibility to directly link ultrastructure with emergent functionality. To improve throughput, micropatterning display approaches can be considered, such as patterning fibrinogen or other biomaterials44. It is also enticing to consider the use of arrayed inertial focussing of platelets in suspension at the same focal height45. Coupled with periodic stop-flow operation to prevent image blurring46, ultrastructure image cytometry has the potential to be undertaken with massive gains in throughput. In summary, the promise of the use of super-resolution microscopy techniques, aided by microfluidic display approaches, will allow the ultrastructural diversity within platelet populations to be more comprehensively characterised.

**2. Platelet Ageing and Priming Impacting Diversity**

Increasing evidence shows that the 'life-long' exposure of circulating platelets to an ever-changing environment influences their activation characteristics, elimination tendency and roles in haemostasis and thrombosis.

***2.1. Biochemical and functional changes arising from platelet ageing***

Under physiological conditions, human platelets have a short, 7–10 days, lifespan that is terminated by apoptotic-like processes37 and by surface glycosylation losses47. Whether each platelet undergoes a gradual or sudden deterioration, is still largely unclear, despite numerous measurements of platelet turnover in knockout mouse models. In disease scenarios, especially linked to thrombocytopenia, there is evidence that prior (immune-, drug- or infection-induced) platelet activation leads to abrupt platelet redistribution and clearance. According to many authors, the age of platelets is considered a major factor impacting their reactivity distribution.

Traditionally, platelet age measurements were achieved by intravenous isotope labelling in baboons, providing the first information that platelet age rather than size determines their reactivity48. In mouse, platelet ageing was found to be accompanied with surface protein desialylation, a process in which locally released neuraminidases can assist49. In human, a similar neuraminidase process is triggered by GPIbα-mediated and αIIbβ3-integrin signalling50. Both desialylation and apoptotic phosphatidylserine exposure trigger hepatocytic capturing of the platelets51.

New studies are welcomed to determine if platelet ageing leads to continuous changes in functionality or to a sudden point of collapse, and if old platelets retain or attain different roles in haemostasis and thrombosis, as well as in vascular inflammation and repair52. To decipher the functional and possibly clinical consequences of platelet ageing, function and composition measurements can be applied to platelet fractions pooled by age markers (*e.g*., glycosylation). More detailed (glyco)proteomic profiles at single platelet resolution can be obtained using sophisticated multi-colour and mass cytometry. The suitability of this approach has already been shown for a Glanzmann patient53. Multi-colour flow cytometry has also revealed the presence of multiple platelet populations in addition to age effects54. Future studies in (immune-thrombocytopenic) patient groups may disclose how young platelets behave in disease-mediated platelet clearance, for instance using emerging liver55 or spleen56 on-a-chip platforms. Overall, the combination of platelet sorting approaches coupled with multi-colour flow cytometry and mass spectrometry (glycoproteomic) profiling and functional assays will provide an in-depth view of the nature and consequences of platelet ageing.

***2.2. Reticulated platelets and functional diversity***

It has long been speculated that juvenile platelets, newly released into the circulation, are more reactive and thus contribute to the risk of cardiovascular disease57. Also known as reticulated or ‘immature’ platelets, the measurement of juvenile platelets is considered to have clinical and diagnostic utility, with their abundance being a combined marker of MK activity and platelet clearance57,58. In mouse, platelet biotinylation and labelling techniques with fluorophores has been used to distinguish juvenile platelets from older ones57,59. Reticulated platelets are named after their ribosome-studded reticulum and have a high mRNA content with a transcriptome of 3–6k mRNAs60. The increased RNA level of human reticulated platelets allow their identification using thiazole orange and 5'Cy5-oligo-dT dyes61. Reticulated platelets have the capacity for mRNA splicing and translation62. While the overall RNA content decreases upon ageing, transcripts occupied by ribosomes appear protected63. This suggests that especially juvenile platelets, with protein synthesis machinery, are capable of functional adaptation. Nevertheless, the links between total RNA level, protein synthesis potential and functional reactivity within a single platelet are unknown.

In order to advance knowledge of platelet age effects in humans, we anticipate experiments involving sorting platelets by age (*e.g.,* by sialylation levels) and linking their functional capacity with their transcriptome and proteome. Ideally, such investigations are undertaken with single platelet resolution, necessitating NGS read-outs for transcripts and CITE-seq for select proteins. The miniscule single-platelet transcriptome (2.2 fg) and proteome represent a sensitivity challenge. Ultrasensitive, targeted approaches such as Constellation-seq64 will be required in which the available NGS read space is dedicated to only transcripts of interest, allowing the detection of low-abundance transcripts. Constellation-seq has not yet been applied to platelets, but promises interesting insights into single platelet heterogeneity. Data sparsity is also confounded by RNA degradation, which will likely require custom bioinformatics for data interpretation. We anticipate that targeted and sensitive NGS read-outs, integrating the transcriptome and proteome of reticulated platelets, will provide a definitive characterisation of this population. Linkage between the molecular make-up and functional assays may then precisely determine the role of the reticulated platelet population in haemostasis and cardiovascular disease. The knowledge gained can also lead to improved assays for platelet phenotyping and diagnosis.

***2.3. Platelet priming as a source of diverse reactivity***

Circulating platelets are exposed to a multitude of environmental cues that can modulate their function, a phenomenon called priming, in which negative priming for example by endothelial cells increases the activation threshold, whereas positive priming in diseased conditions lowers the activation threshold with a greater tendency for thrombus formation1. Such priming can also be achieved *in vitro* by exposing platelets to inhibitors (prostacyclin, nitric oxide) or weak agonists (adrenaline, thrombopoietin)65. Priming alters signalling pathways involved in ligand and shear stress-dependent responses, which can be reproduced in stenosis thrombus formation models66. *Ex vivo* models examining negative or positive priming hold promise for understanding reactivity shifts in platelet populations. Multi-colour and mass cytometry can provide sufficient end-points (20–50x) to better resolve platelet heterogeneity, while CITE-seq gives additional (200) phenotypic end-points. Beyond this, future sensitivity gains in mass spectrometry67 and conceivably nanopore sequencing68 will allow priming related proteome alterations to be characterised at the scale of a few or even single cells.

It is appealing to speculate that juvenile platelets, with a dynamic transcriptome, respond to priming by way of translational adaptation. In some platelet activation scenarios, resident pre-RNA splicing plays a role62 and proteome changes are observed69. This may result in transcriptome remodelling after acute myocardial infarction70. In the near future, we can anticipate priming-induced changes in platelet heterogeneity being determined with single-platelet resolution. For example, the sensitivity of Constellation-seq64 has the potential to be combined with long-read (2 kb) nanopore sequencing71 to capture platelet sub-populations with distinct splice variants. This can be followed by CITE-seq25 to confirm proteomic adaptations. The workings of these NGS-based transcriptome and proteome profiling methods along with spatial multi-omics methods (section 3.1) documented in this review are described in **Figure 3**.

Overall, the integration of new single-platelet data sets capturing differences in signalling pathways, splice variants and associated proteome modifications will provide new insights into how platelet populations arise, have altered functional responses, and contribute to cardiovascular disease.

**3. Intrinsic and Acquired Diversity of Platelets in Thrombus Formation**

Distinct platelet types with different structural, functional and adaptive features are considered to contribute to the heterogeneous organisation of the growing thrombus, with implications for pathological thrombosis. Conversely, the spatiotemporal build-up of a thrombus will contribute to the formation of platelet populations with distinct responses. The precise contribution of these aspects to platelet diversity has yet to be defined.

***3.1. Platelet populations in thrombus organisation and stability***

Conventional flow cytometric approaches have indicated that for almost all surface glycoproteins a 10-fold difference exists between the lower and upper 10 percentiles of platelets from the same donor1. Similarly, flow cytometric evaluation of re-programmed MKs, derived from the same cell clone, surprisingly showed a two orders of magnitude difference in expression levels of GPIbα, GPVI and integrin αIIbβ311. Given these large differences in glycoprotein and receptor make-up, one expects substantial differences between platelets in activation and signalling. It is tentative to assume that platelets that express higher receptor numbers are more active in functional settings, notably in thrombus formation. In this context, it would be interesting to reverse current high throughput screening assays of G-protein coupled receptors, by printing arrays of anti-receptor antibodies and establish the competitive binding of single platelets in combination with activation-dependent imaging.

Single platelet encapsulation in microfluidic droplets has been used to measure intrinsic platelet sensitivity to activation, and demonstrated broad-spectrum variations. Reintroducing paracrine signalling between platelets increased platelet population reactivity twenty-fold72,73. This implies the presence of hyper-reactive platelets with a pioneer haemostatic function, although this remains speculation without experiments linking such platelets to thrombus initiation. With continued thrombus growth, the phosphatidylserine-exposing platelet polarisation phenomena occurs, thus producing pro-aggregatory and pro-coagulant states74. Again using droplet microfluidics this was shown to be an intrinsic divergence program72. However, this still does not explain the zonal distribution of these two phenotypes within the thrombus.

In addition to intrinsic variable reactivity and functional polarisation, high throughput spreading assays have gained insights into the mechanical functional diversity of platelets75 with implications for possible roles in thrombus formation. Similarly, micropatterned fibrin or fibrinogen arrays have measured single platelet contraction forces, enabling the identification of a highly contractile platelet subpopulation which was absent in a subset of patients with undiagnosed bleeding disorders44. Whether this variety is also represented within a platelet plug or thrombus is unknown. The use of mechanical reporters76, such as fluorescent resonant energy transfer (FRET) pairs, allows contraction forces to be measured throughout the entire process of thrombus initiation, growth and arrest. Such measurements will determine whether contractile behaviour is intrinsic or is governed by position and timing within the developing thrombus.

Thrombus initiation is considered to be driven by vessel wall activation, inflammation or injury. Intermediate-throughput assays using microspot arrays of vascular components within microfluidic chambers can be used to produce thrombi with multi-parameter platelet activation measures to assess thrombus build-up and stability from whole-blood. While the thrombi obtained with blood from healthy subjects and patients exhibited some variability, those from patients with platelet function disorders produced distinct thrombus formation patterns39,77. Building on these experiments, endothelialised microvasculature-on-a-chip systems have recently been developed78 and used to incorporate coagulation and fibrin clotting facets in thrombus growth35.

Thrombi formed *in vivo* in mice or *in vitro* with human blood are far from homogenous assemblies, but consist of organised layers of platelets with heterogeneous properties, with patches of aggregating, secreting and procoagulant platelets, distributed over the contracted and fibrin-containing zone of the thrombus core and loosely connected platelets in the outer thrombus shell74,79,80. While the contributing platelets are diverse in properties, whether they are already programmed for diversity as they arrive during thrombus growth remains unknown. The advent of spatial transcriptomics involving *in situ* sequencing81 and the more recent introduction of the DBiT-seq82 (deterministic barcoding in tissue for spatial omics sequencing) method holds exciting promise to provide a comprehensive multi-dimensional map of sections from a thrombus. In DBiT-seq, classical tissue staining and immuno-histochemistry are combined with orthogonal oligo-labelling to provide an array of 10 m pixels for transcriptome measurements, with proteome measurements achieved by integrating CITE-seq25.

Quantitative (phospho)proteome analysis can also impact our understanding of thrombus organisation by, for example, fractionally dissolving the platelets of the outer shell and inner core of a thrombus formed *in vitro*. We foresee that the contribution of intrinsic platelet diversity to a heterogeneous thrombus build-up can be predicted from the responsiveness of single platelets, determined using flow cytometry and droplet microfluidics. Tentatively, NGS-based transcriptome and epitope spatial mapping can help to understand the large-scale platelet interactions governing thrombus growth and stability.

***3.2. Avenues opened by advanced imaging techniques***

Recent milestones in super-resolution imaging can offer new insights into platelet functional diversity. These include the identification of multiple posodome-like actin nodule structures in multiple spread human platelets using a combination of STORM and total internal reflection fluorescence (TIRF) microscopy83; oligonucleotide-tethered single molecule localization microscopy (SMLM) to resolve single-cell contraction forces84; and live measurements of GPVI movements and activation in murine platelet membranes using TIRF with low z-axis depth85. In other fields of cell biology the combination of TIRF and SIM produces 115 nm lateral resolution imaging at 100 Hz capture rates86. These fast and high-resolution imaging methods ideally lend themselves to the challenge of investigating the dynamics of inter-platelet cooperation during thrombus formation, including the spatial heterogeneity induced by the formation of ballooned, procoagulant platelets87. Thus, those platelets initiating thrombus formation and the later platelets trapped in the growing thrombus can be characterised in terms of shape, ultrastructure and behaviour.

Intra-vital microscopy using mouse models has been used to provide a holistic and authentic microenvironment for investigating thrombus formation dynamics. The requirements are ultrafast laser multi-photon excitation to achieve a depth penetration up to 1 mm88. Application of this technique to human platelets in flow in vessel-on-chip models makes it possible to simulate platelet delivery, vessel wall shear and dispersion effects that are relevant to haemostasis, venous and arterial thrombosis scenarios. These dense, multicellular experimental models introduce substantial optical scattering, which can be compensated for by multi-photon excitation34, although this technique has limited z-axis resolution. In contrast, extreme *z*-resolution (10–20 nm) is obtained using reflectance contrast interference microscopy (RCIM), albeit at limited depths (~100 nm), restricting its application to processes such as single platelet spreading89. This highlights the trade-offs when capturing three-dimensional information across nanometer to micrometer length scales.

Fluorescent probes are conventionally used to pin-point molecular dynamics during thrombus formation, but also have their limitations by perturbing platelet biology and causing photobleaching and phototoxicity. Instead, label-free, non-linear imaging approaches do not have these limitations, with second harmonic generation (SGH), for instance, able to capture the distribution of certain collagen fibres90, third harmonic generation (TGH) able to resolve water-lipid/water-protein interfaces (*i.e.* cellular interfaces)34, and coherent anti-Stokes Raman scattering (CARS) allowing biochemical imaging by vibrational selectivity91. These novel super-resolution and non-linear imaging techniques each have their merits and limitations, such that we can anticipate combined multi-modal approaches being used to resolve platelet interactions during thrombus formation in greater detail.

***3.3. Platelet communication diversity in thrombus development***

Activated platelets are famed for rapid and potent paracrine signalling to recruit more platelets to the site of vascular damage92. Ultrastructural analysis revealed different secretion capacities between platelets, in terms of magnitude and types. Microfabricated techniques have been used for single-cell secretome analyses93, with a microchamber compartmentalisation approach allowing >10-plex profiling of 10,000 single cells94. Application of these techniques will allow single platelet intrinsic communication modalities to be captured. In addition, single-cell cytosolic Ca2+ imaging95, ultrastructural analysis96 and dynamic secretion measurements97 can provide insights into the distribution and heterogeneous kinetics of the secretion process. It may even be possible to capture the different types of ageing and activation-induced platelet extracellular vesicles and link these to effector functions of other blood cells98.

Added to these techniques, super-resolution and non-linear imaging approaches hold promise for the real-time investigation of protein and extracellular vesicle shedding. As these sophisticated approaches mature and become more accessible they are set to provide further insights into the ultra-structured, functional organisation of clots and thrombi, with the potential to show how inter-platelet communication deficiencies can destabilise thrombus formation. These advanced imaging methods can be combined with sophisticated vascular models incorporating different cell types to dissect the complex cellular interplays involved in the spatiotemporal coordination of thrombosis and haemostasis, and with extension to immune and inflammation scenarios. Clearly, with our limited understanding of single-platelet secretome diversity, microfluidic profiling methods combined with advanced imaging of vessels-on-a-chip models will expand our knowledge of the interactions of platelets and other cells in thrombus development and thrombo-inflammation.

**4. Future Perspectives and Conclusions**

This review charts the emergence of new, powerful, high resolution and advanced throughput techniques for expanding our understanding of platelet diversity. **Figure 4** summarizes how current techniques have contributed to our knowledge of platelet diversity arising from different sources and highlights the emerging technologies set to extend this knowledge. We foresee NGS-based transcriptome and proteome read-outs in combination with multi-modal ultrastructural imaging and organ-on-a-chip models will provide better insights into the diversity of platelets. In the context of cardiovascular disease, combinations of the new technologies will provide better structural and functional information with single platelet resolution. This will help us to understand if indeed juvenile, reticulated or positively primed platelets influence thrombus formation potential, as well as platelets with higher levels of receptors, signalling proteins and granular contents. On the other hand, the combined technologies may reveal if populations of aged platelets, platelets with a specific set of allele transcripts, and platelets with partial defects in receptors or secretion machinery are less prone to promote thrombosis.

In this review, we have integrated published findings from mouse *in vivo* and human blood *in vitro* studies. Although there are substantial inter-species similarities, it should be noted that there are known differences between human and mouse platelets. Additionally to their smaller size, mouse platelets tend to activate more readily and have a different receptor repertoire to human platelets99. However, a first comparison of the mouse and human platelet transcriptome and proteome shows high resemblance between their signalling proteins, although there are differences in the fine-tuning of signalling cascades100.

The broad-reaching emerging technologies discussed in this review will together create rich, multi-dimensional datasets that will likely be supported by computational approaches to allow better demarcation and characterisation of platelet populations and platelet-derived biomarkers predicting thrombosis risk. In summary, these are exciting times to investigate platelet diversity, with new possibilities of defining novel biomarkers and the rational design of new therapeutic strategies targeting specific platelet subpopulations and behavioural responses now within our reach.

*Acknowledgements.*The authors thank Edyta Wojtowicz (Earlham Institute, Norwich, UK) and Peter Johnson (University of Southampton, UK) for valuable discussions.

*Sources of Funding.* This work is supported by the Landsteiner Foundation for Blood Transfusion Research Grant (Nr. 1711, JH), the British Heart Foundation (FS/13/67/30473, JW) and the Medical Research Council (MC\_PC\_15078, JW).

*Disclosure.* The authors have no conflicts of interest to declare.

*Authors Contributions.* JH and JW conceived and wrote the review together.

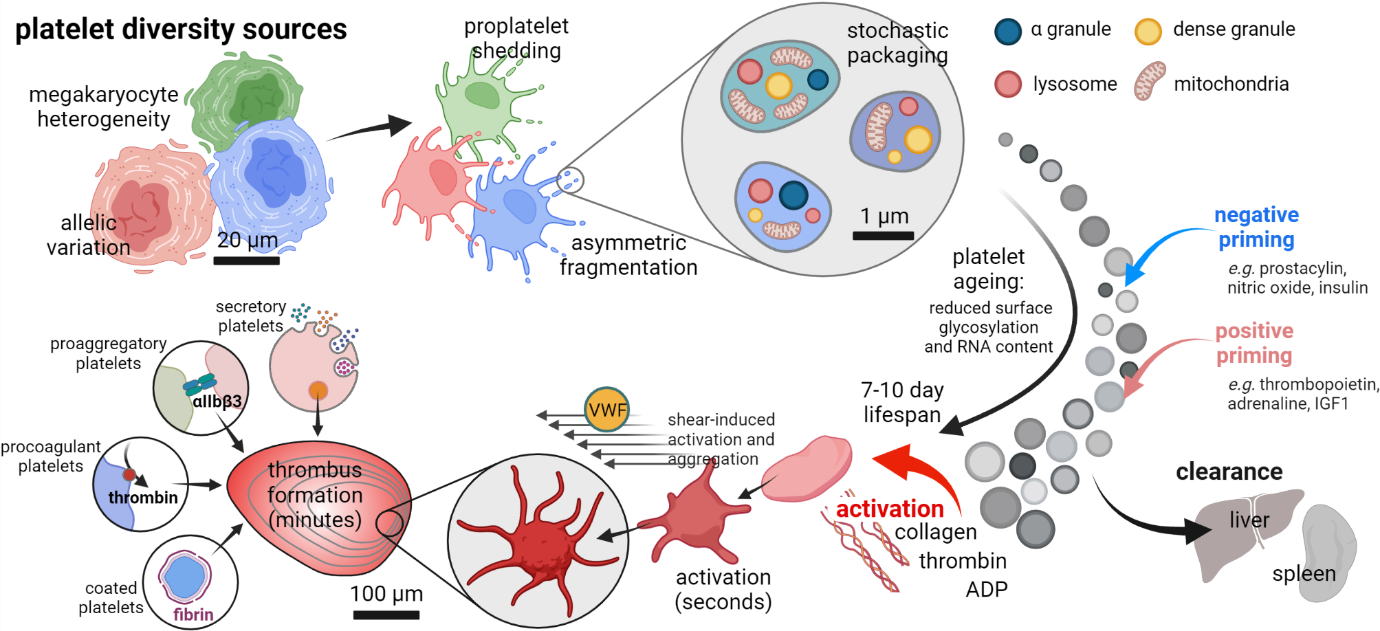
**Highlights**

* Functionally-distinct platelet populations exist with likely different contributions to cardiovascular disease.
* The diversity between platelet populations is not well understood, and advanced molecular and imaging technologies are becoming available to provide new insights.
* Next-generation sequencing read-outs promise to profile the molecular composition of platelet populations, while super-resolution imaging and microfluidic methods can provide new structure-function linkages.
* Integration of new multi-modal and multi-dimensional data sets will greatly expand our understanding of the nature and consequences of platelet diversity, providing biomarkers for thrombosis risk and identifying new therapeutic strategies.

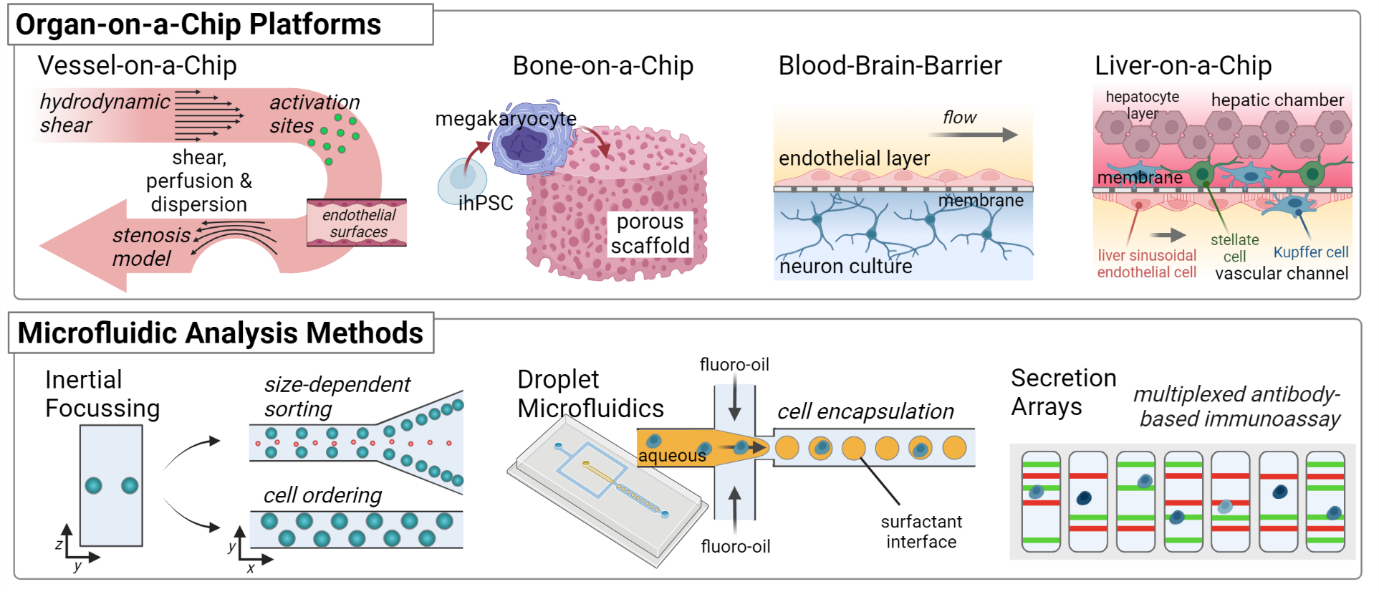
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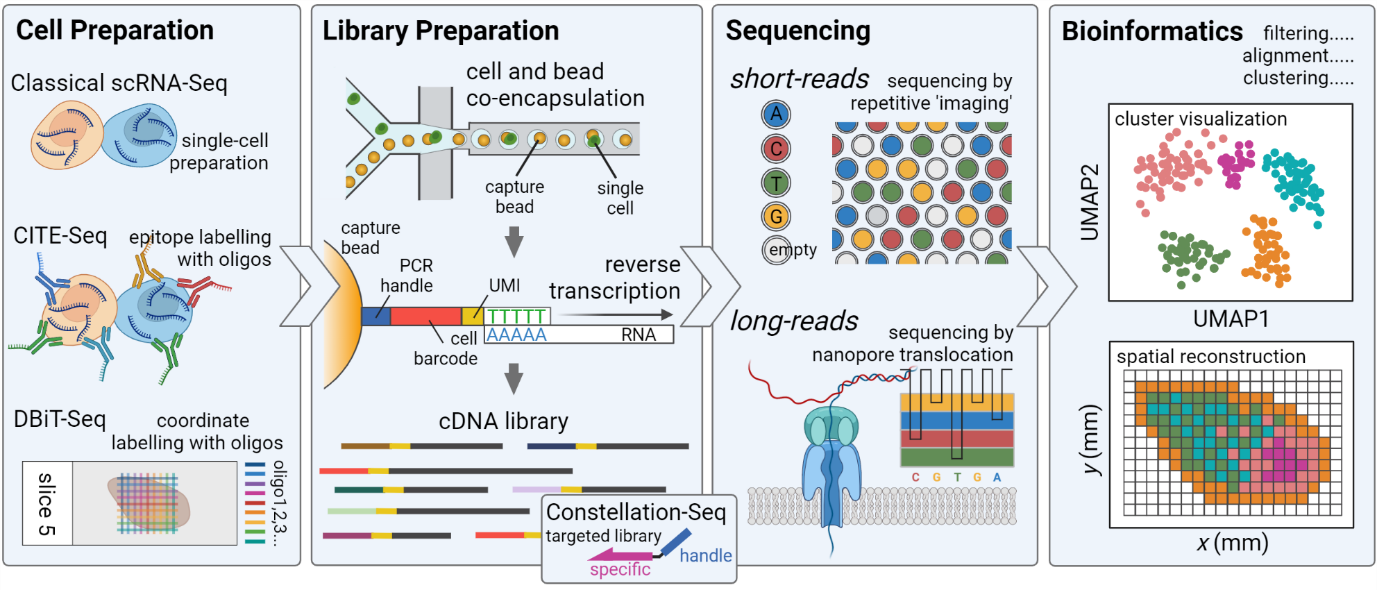
**Figures and Figure Legends**

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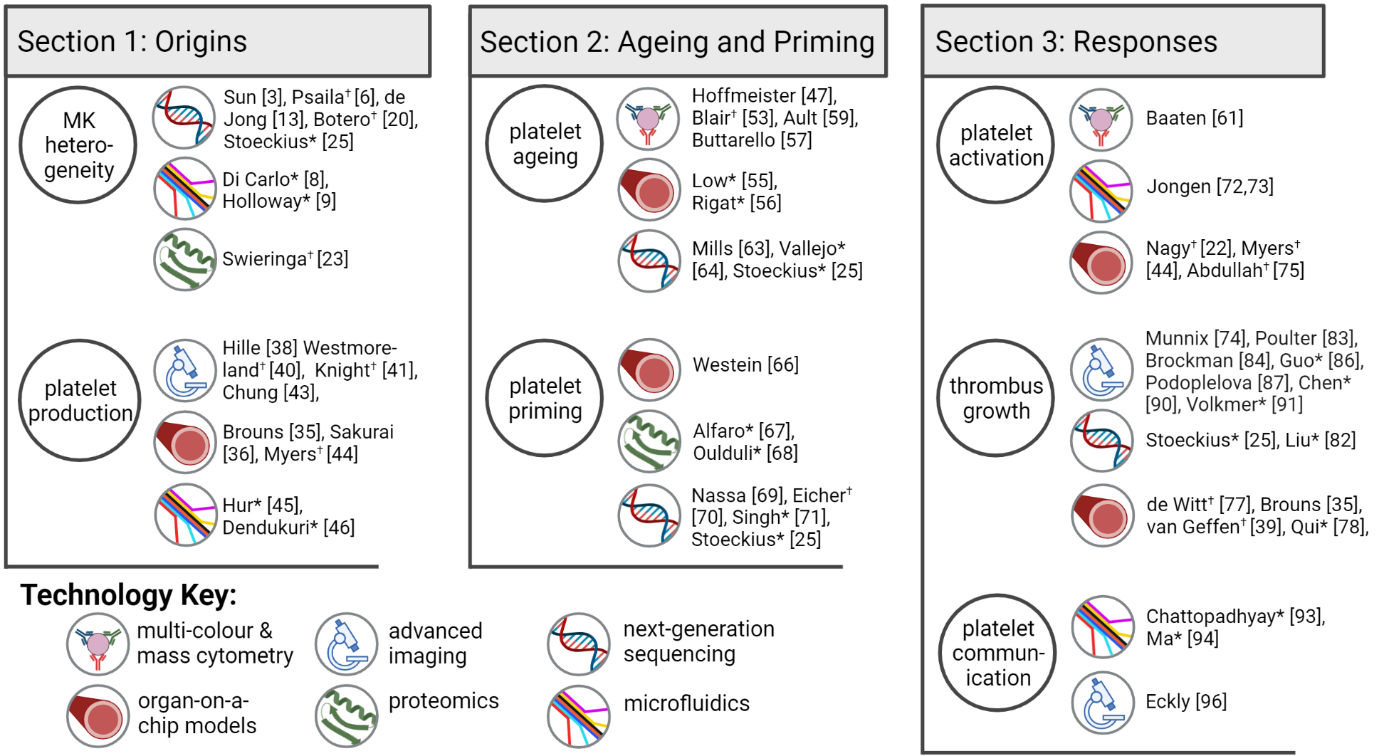
**Figure 1.** **The sources of diversity occurring throughout the ‘lifespan’ of platelets.** Clockwise this review is structured around the origins of platelet diversity (section 1), diversity arising from ageing and priming (section 2) and diverse responses during thrombus formation (section 3): The contribution of heterogeneous megakaryocytes to platelet diversity is explored (Section 1.1), followed by the consequences of megakaryocyte allelic variation (Section 1.2), and the asymmetric process of proplatelet shedding and further fragmentation, and how this causes ultrastructural variation in platelets (Section 1.3). Building on these origins, we discuss the possibility of age defining functionally distinct platelet populations (Section 2.1), and then focus on the so-called juvenile or reticulated platelets (Section 2.2). In addition, environmental adaptation through positive or negative priming provides a source of platelet diversity (Section 2.3). How different platelets contribute to heterogeneous spatiotemporal diversification during thrombus formation is then discussed (Section 3.1). Advanced imaging techniques promise an improved understanding of the dynamics of thrombus development with populations of secretory, pro-aggregatory, procoagulant and coated platelet populations (Section 3.2). Platelet communication may prove to be important in this setting, requiring knowledge of singe platelet secretomes (Section 3.3). Created with BioRender.com.



**Figure 2. Organ-on-a-chip platforms versus microfluidic analysis methods**. Organ-on-a-chip platforms aim to recapitulate physiological niches *ex vivo*. Relevant to platelet biology, vessel-on-a-chip models capture flow-induced perfusion, dispersion and shear effects. Stenotic flows and activation sites can be used to mimic thrombus development, and surface endothelialisation provides a more complex representation of vessel physiology. Bone-on-a-chip platforms typically involve the use of porous scaffolds for providing a culture niche for megakaryocytes, with human models comprising human induced pluripotent stem cells (hiPSCs). Blood-brain-barrier and liver-on-a-chip platforms typically adopt membranes to interface co-cultures while permitting flow within a vascular channel coated with endothelial cells. In contrast, microfluidic methods aim to introduce analytical functionality. Relevant to this review, inertial cell focussing has the potential for sorting megakaryocytes, and ordering platelets for high throughput imaging. Droplet microfluidics lends itself to capturing intrinsic platelet functionality (without paracrine cross-talk). Alternatively, nanowell arrays can be used to capture single cells for interfacing with surface-patterned antibody stripes to allow secretions to be profiled using immunoassays. Created with BioRender.com.



**Figure 3. Single-cell next-generation sequencing workflows.** NGS methods have the potential for profiling platelet diversity and involve various cell preparation methods, followed by cDNA library preparation, sequencing and bioinformatics for data interpretation: Classical scRNA-Seq involves preparing a single-cell suspension. In addition, oligo-labelled antibodies can be used to identify epitopes in combination with the transcriptome by CITE-Seq, and spatial information can be obtained by the orthogonal labelling of cells in a tissue section prior to cell disaggregation by an overall workflow called DBiT-Seq. The cell suspension is then typically encapsulated in droplets with mRNA capture beads (*e.g.* 10X Chromium), followed by a reverse transcription step to create a cDNA library with a unique barcode linking to each cell and a unique molecular identifier (UMI) for transcript quantification. The small platelet transcriptome requires ultra-sensitive approaches such as Constellation-Seq in which a targeted, linear amplification approach is used to capture transcripts of interest. Libraries are then subject to sequencing, with short-reads (~100 bp) commonly obtained by repetitive multi-colour imaging (Illumina) and long-reads (≤2kb) obtained by current-based nanopore sequencing (Oxford Nanopore Technologies). Sophisticated bioinformatics routines are required to filter, align and cluster the single-cell data, with clusters visualized using a UMAP (uniform manifold approximation and projection) plot. Cell clusters can also be spatially reconstructed using coordinate sequences from the DBiT-Seq process or from the 10X Visium platform. Created with BioRender.com.MK



**Figure 4.** Advanced technologies for investigating platelet diversity classified by type and linked to major questions and associated sections of this review. References with a † have demonstrated clinical potential, while those with a \* have yet to be applied to platelet research. Created with BioRender.com.