

### Microfluidic single-cell analysis for systems immunology

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The immune system constantly battles infection and tissue damage, but exaggerated immune responses lead to allergies, autoimmunity and cancer. Discrimination of self from foreign and the fine-tuning of immunity are achieved by information processing pathways, whose regulatory mechanisms are little understood. Cell-to-cell variability and stochastic molecular interactions result in diverse cellular responses to identical signaling inputs, casting doubt on the reliability of traditional population-averaged analyses. Furthermore, dynamic molecular and cellular interactions create emergent properties that change over multiple time scales. Understanding immunity in the face of complexity and noisy dynamics requires time-dependent analysis of single-cells in a proper context. Microfluidic systems create precisely defined microenvironments by controlling fluidic and surface chemistries, feature sizes, geometries and signal input timing, and thus enable quantitative multi-parameter analysis of single cells. Such qualities allow observable dynamic environments approaching *in vivo* levels of biological complexity. Seamless parallelization of functional units in microfluidic devices allows high-throughput measurements, an essential feature for statistically meaningful analysis of naturally variable biological systems. These abilities recapitulate diverse scenarios such as cell-cell signaling, migration, differentiation, antibody and cytokine production, clonal selection, and cell lysis, thereby enabling accurate and meaningful study of immune behaviors *in vitro*.

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### Introduction

The immune system protects organisms from a vastly variable set of challenges, ranging from sterile injury to attacks by pathogens, and achieves a specific, fine-tuned response. A

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research interests focus on applying advanced microfluidics to study systems biology, as well as developing new methods of micro and nanofabrication to improve capabilities of *in vitro* cell culture.

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*developing high-throughput microfluidic and optofluidic technologies, and using them to decipher cellular information processing. The Tay Lab is particularly interested in spatiotemporal analysis of immune pathways, cytokine secretion, stem-cell differentiation, and cell migration, all with single-cell resolution.*

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dynamic and interactive set of biomolecules, cell types, organs, and behaviors are mobilized to accomplish this daunting task. These parts constitute a system coordinating its action over varying time and length scales. These multi-level interactions are increasingly being recognized as fundamental to the proper functioning of many biological processes. The notion that the whole is bigger than the sum of its parts is especially true for the immune system, which exhibits complex emergent properties for every aspect that has been studied. Thus, the study of immunity at the systems level is imperative to fully understand it, and to eventually build predictive models. The need for such understanding and models becomes clear considering the number of immune related diseases that do not have viable treatments. Conditions such as sepsis, HIV, tuberculosis, malaria, hepatitis C and others such as autoimmune dysfunctions and even the common cold are intensely studied, but remain major health issues. Limited knowledge of the immune system directly results in a high level of global mortality and morbidity. This lack of knowledge stems from the complexity of each condition and their constantly changing temporal nature, placing severe limitations on their study and discovery of treatments.

Systems-level investigations can add fundamentally to understanding such ailments and to uncovering treatments for these conditions.<sup>1–5</sup> Microfluidic systems<sup>6</sup> provide a direct and powerful means to meet challenges inherent to understanding immunity and resolving global health concerns. This review will discuss the microfluidic methods developed to this end, and describe how they have been implemented for the study of immunity.

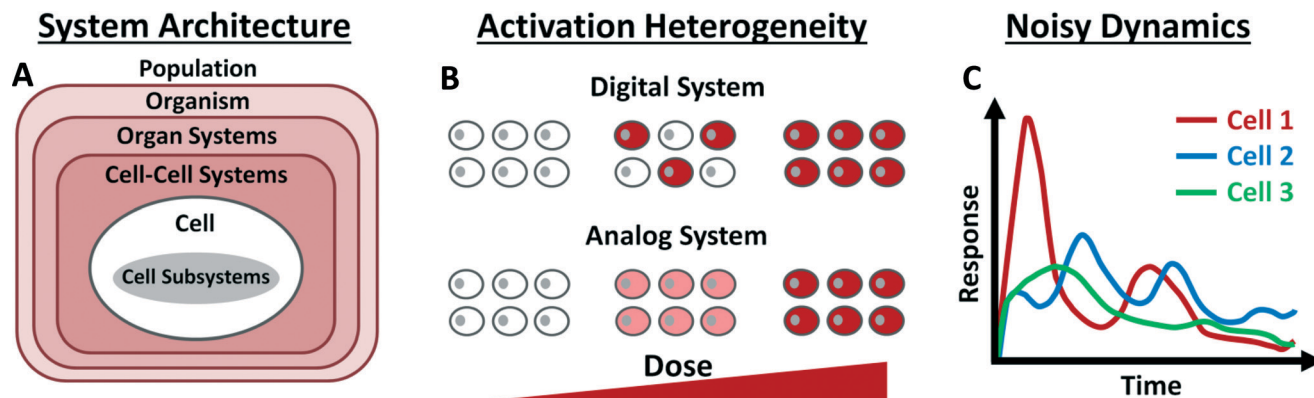
The systems-level interconnected nature of the immune response can be appreciated considering a basic infection scenario comprising a host of steps leading to successful bodily protection.<sup>7</sup> It begins with cellular pathogen detection by pattern recognition receptors, activation of innate immune pathways, and subsequent soluble molecule signaling to other cells. Signaling triggers immune cell migration to the infection site. This is followed by confrontation of the invading organism for local destruction. Complimentary processing of pathogen components by antigen presenting cells transports antigens to lymph nodes. There, adaptive immune cells that recognize their cognate antigen are activated, proliferate, and aid in clearing invaders. Antibody producing cells also proliferate and release various classes of antibodies to assist in pathogen clearance and to retain memory of the attack. Eventually this cascade subsides, and the immune system returns to a resting state. Conversely, a nonresolving, chronic immune response may lead to several deleterious conditions such as autoimmune disease, allergy, and cancer. Post-resolution, a population of cells is set aside to serve as a form of memory to fight the disease if reencountered. During this process, multiple cell types communicate with each other *via* soluble chemicals and cell surface receptor–ligand pairs to coordinate, reinforce, and modulate their functions. Without these interactions, an effective and lasting defense cannot be mounted. A high degree of complexity is apparent

even in such a simplified scenario, and operation of the system as a whole consists of many subsystems exercising individual functions, but relying on and communicating with other systems and levels of organization. The challenge then is apparent: how to study the whole immune system, and not just its parts. Much can and has been learned by reductive approaches studying individual cell types and behaviors, but systems-level investigations of immunity are still lacking due to technical challenges.

A fundamental subsystem of immune operation is the single immune cell, with multiple varieties of immune cells carrying out the various functions of the immune system. These single cells may function as lone operators, but also coordinate with other immune cells and tissue. Behavior at this level, however, poses unique challenges for understanding immunity. Central to this is that in any biological system, there exists a large degree of noise. Forms of biological noise including both environmental (*i.e.* different signaling histories or cellular states) and internal sources (*i.e.* stochastic molecular dynamics) are present. Cells have built-in noise reduction mechanisms and variability can be minimized experimentally, however noise can never be completely eliminated. This ultimately manifests as heterogeneous single-cell behavior, where cells exposed to identical inputs react in different manners. This diversity of function is ever present and is a fundamental and likely essential aspect of cell biology.<sup>8</sup> Accordingly, variation at this level must be accounted for, and cells should be measured individually. Bulk (population-averaged) measurements which combine data from multiple cells will result in masking cellular diversity. Thus pooled measurements will often lead to incorrect and incomplete understanding of functions.<sup>9</sup>

Also characteristic to immunity, and cell biology in general, is its dynamic nature. Disease and injury must be rapidly dealt with to ensure organism health and survival. This challenge is met at several levels of innate and adaptive immunity. These levels of defense each have characteristic time scales governing effective expression of their specific roles. For innate immunity, initial reactions must recognize a threat and signal to additional cell types for assistance. These cells then combat the threat before eventually returning to a resting state or undergoing apoptosis. For adaptive immunity, antibody and cell receptor production must be coordinated with foreign antigen detections before proliferation. After proliferation and defense, these cells will for the most part undergo apoptosis. Countless other time dependent interactions exist inside a system that is in a state of continuous flux. The result is temporally varying behavior across many cell types. Again, comparing to standard bulk, or single time point measurements, (such as those obtained by staining, western blots, PCR, Boyden chambers, ELISA, ELISPOT, microarrays, or flow cytometry), single-cell dynamics should be measured or significant aspects of behavior will be omitted.

Considering challenges posed by the nature of the immune system, Fig. 1 can guide development of measurement tools



**Fig. 1** Challenges characteristic to the study of immunity. (A) Immune functions cross various levels of organization and subsystems, which are coupled through dynamical interactions. (B) Responses to immune signals are heterogeneous, necessitating single-cell analysis. Analysis of individual cells can distinguish between digital and analogue immune response (top and bottom rows, respectively). Resting cells (in white) can become activated in a digital way, where increasing dose leads to an increased fraction of fully activated cells (red). In an analogue system, increasing dose leads to gradually increasing response intensity, from inactive (white), to partially active (pink) and to fully active (red). Population-averaged bulk measurements can not distinguish such a difference. (C) Temporal characteristics of individual cells can differ significantly and exhibit noisy dynamics, necessitating high-resolution quantitative analysis of single-cells over time.

to effectively study it. Generally, dynamic, multiparameter, single-cell measurements are expected from such analytical tools. Such measurements should also be highly quantitative and high-throughput in nature to become useful in computational modeling studies. This should extend beyond single parameters to encompass multiple measurements covering diverse aspects of cell behavior. These include signaling state, mRNA and protein expression, secretion, migration, cell-cell interactions and others measured on the same cell. Such multi-dimensional analysis will reveal an overall picture of the many parameters comprising a given cell state and behavior.

Coupled with a need for an expansive parameter space is the need for experimental control. Creation of a predesigned microenvironment as well as ability to temporally change conditions allows meaningful recapitulation of *in vivo* conditions. Many spatial cues such as surface and solution chemistry, and cell-cell interactions are determining factors in cell behavior and need to be modifiable. Proper timing and sequence of signaling events are crucial in many immune functions, and so temporal control should be possible in an ideal experimental setup. Spatial and temporal control must therefore be under direct, programmable system regulation. This allows precisely timed perturbation of conditions, can provide feedback, and will allow analyzing basic dynamic aspects of immune behavior.

Creation of microfluidic systems incorporating the aspects discussed above involves increasing levels of complexity compared to standard techniques and thus would benefit from automation and streamlining. Increased ease of use, robust and user friendly chip-to-lab interfacing, compact design, and automated data recording and analysis are also useful features in a typical biological research setting. Additionally, some requirements may conflict considering abilities of current technologies, or may need to be achieved off chip. While no single microfluidic system will comprise an ideal device, there has been a trend towards realizing many of these

challenging goals with microfluidic systems. In this review, the recent developments using microfluidics to study immune system function will be discussed. Applications have been separated according to basic immune functions addressed by each study, including: immune activation, secretion of signaling molecules, cell migration, cell removal, antibody production, and the area of drug screening. Related areas of study such as pathogen behavior, cancer, and microfluidic point-of-care diagnostic tools and sensors are not covered by this review with the focus instead centered upon direct, single-cell microfluidic investigations of immunity. A brief discussion of the outlook for future microfluidic study of immunity is also presented.

## Activation of signaling pathways in immune cells

During the course of an immune reaction, a cell encounters an initiating signal and must change its behavior to meet the immune challenge. Observing and quantifying this response is crucial to understanding how the immune system reacts to signals and initiates protective functions. Initiating signals range from innate and adaptive signals such as pathogens, damage signals, to cytokines, antibodies, or other immune cells. These provoke a response and alter behavior in the cell that encounters them. The response eventually takes many forms of external action, but is firstly an internal phenomenon. Reactions are heterogeneous, and do not necessarily agree with population level measurements.<sup>9–13</sup> Investigating this fundamental fact is necessary to understand immunity but is difficult with conventional systems studying large, pooled populations. Moreover, both the signals encountered and the responses are diverse and dynamic. Immune cells can present additional challenges for study such as being non-adherent and requiring extended periods of observation to see a full behavioral range. Microfluidic systems can

address these issues by providing relevant signals as well as detecting the complex changes resulting from immune activation. Moreover, they can do this at a single-cell level addressing natural heterogeneity, with fine time resolution. These advantageous characteristics are relevant to many areas of immunity, and many systems combine observation of immune activation with assays measuring additional downstream actions.

A principle means to observe cell activation is through observing transcription factors. Activation induced transcription factor changes alter cell behaviors, and drive resulting cell reactions. Fluorescent detection *via* staining or genetically encoded fusion proteins allow this to be seen and quantified through fluorescence time-lapse microscopy. Such a method was used by members of our group to see activation and dynamics of the master immune regulator NF- $\kappa$ B by tracking its movement into and out of the nucleus during stimulation with the inflammatory signaling molecule TNF- $\alpha$ .<sup>9</sup> This observation was coupled with a multi-chamber microfluidic chip to repeatedly and controllably stimulate cells at timescales not possible with conventional assays.<sup>14</sup> With this system it was demonstrated that NF- $\kappa$ B activation is fundamentally digital in nature, something that could not be detected when looking at population measurements. The percentage of cells fully responding to TNF- $\alpha$  was seen to increase with an increasing concentration of TNF- $\alpha$ , as in Fig. 1B. The cells responded in a digital, (all or none) fashion. The analog activation response previously observed from bulk measurements was attributed to the increasing percentage of single cells responding fully, and not a population responding in a gradual (analogue) way. Others, such as Awwad *et al.*,<sup>15</sup> have subsequently seen a similar digital response. Their system incorporated cells growing inside microfluidic channels exposed to gradients of stimulants. This system likewise detected the digital NF- $\kappa$ B response of cells stimulated with different cytokine concentrations, in this case of IL-1 $\beta$ .

Heterogeneous activation has also been observed with a variety of other platforms. Activation of single CD4<sup>+</sup> T-cells was studied by Zaretsky and Polonsky *et al.* with a microwell array combined with fluorescent reporting of the regulatory T-cell transcription factor Foxp3 and surface staining of CD69.<sup>16</sup> Confinement provided by microwells allowed repeated observation of these non-adherent cells after activation. The activation measurements detected heterogeneous, single-cell differentiation, and further heterogeneous responses. These showed single-cell differences in apoptosis, suppression and proliferation, as well as daughter cell synchronization.

Diverse pathways are involved in immune reactions and these may not all have readily available fusion proteins. This has been addressed by systems using fluorescent staining for a variety of internal factors such as those by Cheong and Wang *et al.*<sup>17</sup> and by similar work from Srivastava *et al.*<sup>18</sup> and Wu *et al.*<sup>19</sup> The latter works developed systems for detecting activation *via* ERK1/2, p38, TLR4-MD2, and intracellular TNF- $\alpha$  in LPS exposed macrophages. In these systems, cells

were cultured inside microchannels before stimulation and staining. The cells could also be imaged in place, for example, by observing a NF- $\kappa$ B fusion protein. Post-stimulation, cells were detached and assayed in a manner similar to a flow cytometer as they were washed out of the chip. This flow-based stimulation allowed for rapid time point measurements of activation, as fixing compounds could be quickly introduced to stop biochemical reactions after stimulation. Reaction times as fast as five seconds were measured for several thousand cells, though the system does not allow for repeated imaging of the same cells for all components.

Another method for detecting immune activation was developed by Gossett and Tse *et al.*<sup>20</sup> for observing unattached cells flowing through microchannels. Cells were imaged undergoing flow shearing, which detected deformation under different activation states. This simple and label-free method examined activated peripheral blood mononuclear cells (PBMCs). By observing changes in cell shape and deformability, activation could be detected at a rate of up to 2000 cells per second.

Detecting activation is also possible by observing internal calcium ion levels of stimulated cells. Calcium is an important second messenger and its release can trigger a wide range of signaling cascades.<sup>21</sup> A method using single-cell manipulation and activation based on dielectrophoretic capture of cells and beads was used by Kirschbaum *et al.*<sup>22</sup> to probe activation of T-cells. Electrodes captured cells and beads functionalized with anti-CD3 and anti-CD28 antibodies. Cells and beads were subsequently brought together to activate cells while fluorescently examining Ca<sup>2+</sup> levels. Cells were then removed from the chip, grown in well plates, and stained for surface markers indicating T-cell activation. Activation levels were measured and correlated back to single-cell calcium levels seen on chip. This analysis uncovered relationships between initial activation and downstream changes in cell behavior such as that seen between the shape of the Ca<sup>2+</sup> wave and subsequent surface expression of CD69.

Bao *et al.* also looked at calcium activity of immune cells, but used their system to determine sources of inherent noise in the G-protein-coupled calcium response.<sup>23</sup> Their system supplied pulses of stimulants to RAW 264.7 macrophages being fluorescently imaged for calcium release. Sources of inherent noise were seen to stem mainly from long term variations in protein level or protein-protein interactions, and not from short term stochastic influences. The microfluidic setup facilitated this by providing closely spaced, repeated stimuli pulses and detecting high correlation between pulses. The system could additionally track daughter cells and see Ca<sup>2+</sup> response correlations. This gave further credence to long-term influences of variability on calcium response. Calcium-based activation was also observed using an array of cell traps in a method developed by Faley *et al.*<sup>24</sup> Primary T-cells and dendritic cells were hydrodynamically isolated together, or separately, in a trap array prior to stimulations. This allowed hundreds of single T-cells undergoing dendritic cell dependent activation to be observed. They saw



contact dependent T-cell activation and could separate contact *versus* non-contact activation modes.

Another fundamental aspect of the activation induced immune response is differentiation. Differentiation allows cells to take on different functions to meet immune challenges. Changes span from initial differentiation of progenitor cells to mature immune cells, to more nuanced fate decisions of adaptive immune responses such as antibody maturation and selection of different T-cell types. Complex signals drive these changes, which also may have extended time spans. Microfluidic approaches assist study of these phenomena by providing complex stimulation environments and possessing the capability to reliably track individual cells for long time periods.

Culture of cells using commercially available 50  $\mu\text{m}$  microwells was used by Duffy *et al.* to investigate B-cell maturation and antibody isotype switching.<sup>25</sup> The microwells, were used to separate B-cells for observation under stimulation mimicking T-cell interaction. The separation generated by microstructures provided a means to track single-cell divisions in a large number of cells to correlate single-cell behavior to the population activity. This basic method in conjunction with modeling highlighted the inherent random potential of plasmablasts to take on different phenotypes and display different proliferation abilities in an autonomous and stochastic manner. This showed how random individual cell decisions are used to reliably produce a consistent population of B-cells with diverse cell fates that is necessary for proper B-cell function.

Additional systems have incorporated the possibility to conduct several other assays within their systems. Multi-parameter abilities such as these potentially increase the overall measurement space and provide a more complete picture of activation. These consisted of microwell based systems such as those developed by Zurgil *et al.*,<sup>26</sup> Day *et al.*,<sup>27</sup> and Schiffrbauer and Kalma *et al.*<sup>28</sup> which were designed to capture and analyze single cells. These flexible systems consisted of open microwells fabricated from a UV cured polymer, PDMS, or consisted of a cartridge system. These designs were compatible with stimulating and detecting reactive oxygen species (ROS), calcium ions, enzymatic activity, mitochondrial membrane potential, cell-cell lysis, apoptosis immunosynapse formation, and proliferation.

Many other diverse aspects make up immune activation, and promising opportunities remain for their study. These could include monitoring protein-protein interactions, as well as protein abundance and localization,<sup>29,30</sup> gene expression analyses,<sup>31</sup> receptor dynamics, and metabolomics,<sup>32</sup> among others. If immunologically focused studies of these phenomena can be realized, much useful and broad-ranging knowledge of immune activation could be uncovered.

Immune activation is a diverse and dynamic phenomenon, and microfluidic platforms enable improved study of its characteristics in several ways. Central to these is single-cell resolution, which uncovered heterogeneous cell behaviors. This was coupled with dynamic measurements and

multicomponent readouts. This larger, more complete time-based picture of activation is highly relevant for deciphering systems-level cell biology and overcomes limitations of conventional measurement systems.

## Secretion of signaling molecules

After cells have detected an immune stimulus, they coordinate their newly activated functions as part of their response. Signaling *via* soluble molecules such as cytokines and chemokines, reactive oxide species, prostaglandins, and others is employed to mediate immune function between cells as well as attack cells that have been identified as targets. These functions, if not properly directed, can lead to either too little defense, or to a damaging overreaction. Secreted factors guide the course of immune reaction from initial activation and migration, to adaptive immunity, protect against excessive immune response, and eventually to resolution of inflammation. These diverse actions may be exercised by the same cell at different times exhibiting a level of multifunctionality, complicating understanding of secretion. This level of complexity of molecular release and its importance to immune functions has consequently received recent attention *via* microfluidics. Secretion occurs at the level of individual cells which is reflected in the nature of many investigations. Larger, coordinated behavior and communication, however, have also been analyzed to understand existing higher-level coordination. Many methods have used small volumes to detect secreted molecules under various scenarios. This allows for a detection limit to be achieved that matches current assay capabilities, and permits examining single-cell secretion.

The DNA barcode technique developed by the Heath group is one example of small volume containment, wherein single cells are confined inside chambers functionalized with lines of capture antibodies. Around 1000 single cells can be confined inside nanoliter sized chambers possessing ~10–20 stripes of capture antibodies. Cell secreted factors are captured on stripes and spatial separation allows for multiple secretions to be simultaneously detected *via* a subsequent fluorescent sandwich assay, Fig. 2A. This method was used by Shin *et al.* to analyze secretion of stimulated human macrophages.<sup>33</sup> Correlations of different secreted molecules by activated immune cells, and correlation changes were examined in the context of several factors. These included presence of multiple cells inside a chamber, or effects of antibody neutralization on secretion. Analysis led to uncovering characteristics of the system such as why certain cytokines have higher fluctuation levels than others. Ma *et al.* also used a barcode array for profiling multiple secretions of primary, tumor associated human T-cells.<sup>34</sup> They observed that tumor associated T-cells, as compared to T-cells from healthy patients exist in a more active secretory state and had higher functional diversity in terms of kinds of molecules secreted. Additional use of a barcode array was demonstrated by Lu *et al.*<sup>35</sup> to profile primary tumor cell

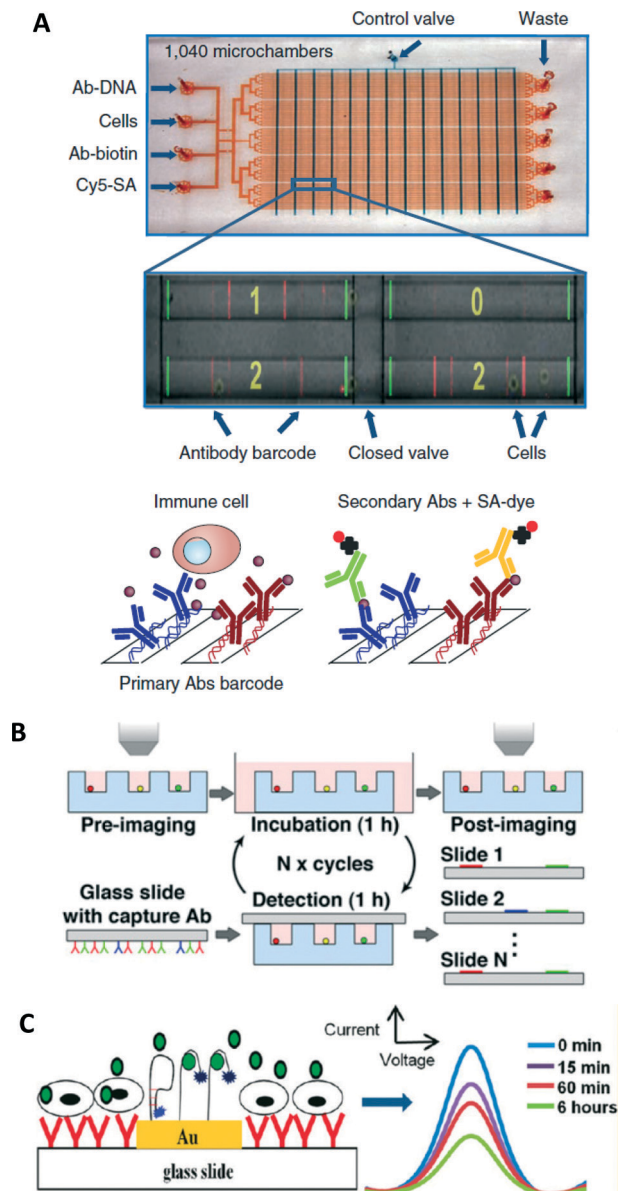


Fig. 2 Detecting immune cell secreted molecules. A) Barcode method. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Med.*,<sup>34</sup> copyright (2011) B) Microwell method<sup>37</sup> C) detection via aptamer functionalized electrodes. Reprinted with permission from ref. 50. Copyright (2011) American Chemical Society.

secretions along with migration. This again showed heterogeneous secretion among a population of similar cells.

An alternative method for confining single cells is the microengraving technique where cells are loaded into a microwell array. These wells typically range in size from 30–50  $\mu\text{m}$ , and are sized so as to capture single cells, Fig. 2B. Once loaded, secreted molecules are captured *via* antibodies attached to the lid closing the well array. The lid is manually removed at different time points and a fluorescent assay conducted on it to determine secretion. The removable lid and open design also usefully allows for simple recovery of screened cells for further growth and analysis. This method

can stochastically capture large numbers of single cells, it assays up to ~four cytokines on up to 25 000 cells and is capable of measuring secreted proteins for several two hour time points. Like the barcode array and other methods, some chambers will be empty or filled with multiple cells due to loading statistics. However, due to the high number of wells in the arrays, sufficient numbers of single cells can be captured. Additionally, simultaneously examining single *versus* multiple cells can provide useful additional data. Chemical inputs to cells must be delivered manually, which limits precision and the ability of establishing temporally complex input profiles such as single-pulses or pulse trains. Additionally, open well designs create possible cross-well contamination issues.<sup>36</sup> Improvements to these microwell systems could include their integration with microfluidic multiplexors and membrane valves to improve system controllability, which could also render them suitable for delivering dynamic inputs to cells.

This method has been applied to a variety of different uses coupling secretion measurement to other aspects of cell activity and human health conditions. These included uncovering sequential, or one-at-a-time release of cytokines by T-cells,<sup>37</sup> simultaneous study of secretion from differently tagged cell types on one array,<sup>38</sup> and cellular secretion as it relates to immunological binding and activation, such that mimicking antigen presenting cells.<sup>39,40</sup> Yet further research using this method has studied T-cell secretion from HIV-infected patients in conjunction with cellular lytic ability,<sup>41</sup> and lysis by natural killer cells in contact with cancer cell line target cells.<sup>42</sup> Finally an assay exposed clinical CD8+ T-cells from HIV+ patients to a range of HIV antigens. This detected secretory responses of each cell to each HIV antigen. Cells responsive to a specific epitope could then be recovered, expanded, and further studied to provide insight into methods for combating HIV.<sup>43</sup>

An alternative commonly used method of cell isolation is by droplet encapsulation. For secretion analysis, Konry *et al.* developed a method using water in oil droplets as a means to isolate primary regulatory T-cells cells.<sup>44</sup> All components including cells, antibody coated capture beads and detection antibodies were encapsulated with single cells in droplets. IL-10 secretion was then detected by localized fluorescence on the surface of the bead. This allowed stimulated single T-cell secreted molecules to be automatically captured and detected in a droplet format. Another droplet encapsulation method was developed by Chokkalingam and Tel *et al.* who incorporated activated Jurkat cells and capture beads into droplets primed with a gelling solution.<sup>45</sup> The droplets were incubated while cells secreted, and subsequent gelation allowed beads and cells to be stained, washed and interrogated *via* flow cytometry. This uncovered several secretory subpopulations of IL-2, TNF- $\alpha$ , and IFN- $\gamma$  released by several thousand cells per experiment.

Other methods have relied on a diverse set of techniques to examine cell secretions. Microfluidic chips for immunophenotyping were created by Huang and Chen for example,

to simplify secretion assays.<sup>46,47</sup> These chips employed two chambers separated by a membrane which allowed for mono-cyte seeding and stimulation to be independent. They used a bead-based ALPHALISA assay<sup>48</sup> conducted in the lower chamber for secretion detection. This system provided a platform for a rapid stimulation and no-wash assay of a low number of cells in a shortened amount of time.

Zhu *et al.*<sup>49</sup> also developed a method combining selective cell capture with secretion measurements. Purified blood was flowed into a chip and anti-CD4 antibodies on spots selectively captured T-cells while washing out nontarget cells. Cytokine specific antibodies on spots then captured cell secreted molecules from immobilized and stimulated cells. This correlated cell secretions with the specifically purified cell types inside the microchannel.

Many of the above described methods measure high numbers of cells and often with multiple time points. High throughput, sensitive, multiple, and real time secretion measurements of single cells, however, would be an ideal tool to unravel secretion behavior under different conditions. While this goal is yet to be realized, work towards this aim is being conducted. Liu *et al.*<sup>50,51</sup> have interestingly developed systems approaching real time monitoring of cytokine secretion from a population of as little as a few hundred cells. They used aptamer coated electrodes as redox sensors which change configuration relative to electrode surface upon cytokine binding, Fig. 2C. This conformational shift detected IFN- $\gamma$  and TNF- $\alpha$  released from activated immune cells at several time points.

Beyond antibodies and cytokines, the immune system response includes secretion of a wide array of substances such as other proteins, enzymes and reactive oxygen species.<sup>52</sup> Electrode based methods have also been used to investigate some of these behaviors such as by Yan *et al.* who developed a method to detect H<sub>2</sub>O<sub>2</sub> secretion from activated macrophages.<sup>53</sup> In this work, horseradish peroxidase contact with H<sub>2</sub>O<sub>2</sub> amplified an electrode signal to detect secreted molecules from hundreds of cells. Others have published a method describing internal nitric oxide production and upregulation of nitric oxide synthases (NOS) though not of secreted nitric oxide (NO).<sup>54</sup> This method relied on single-cell capture inside glass microwells, with an internal fluorescent assay to detect internal NO. Cells were then stained for iNOS to detect correlation of actual synthesis with enzymatic production at the single-cell level.

Microfluidic investigation of secretion has provided interesting insight into this important area of immune function. A variety of systems observed aspects such as the heterogeneous and dynamic character of secretion and its relation to other parameters. These parameters such as internal (transcriptional) activation state, lysis, cell contact, cell type and others were seen in their context of influencing secretory behavior. Some methods additionally captured and examined primary cells, increasing experimental relevance. Recovery of functionally screened cells for further analysis or expansion in culture was also often possible. Undoubtedly, microfluidics

will continue to play an important part in measuring systems-level immune secretory behavior, due to its ability to resolve dynamic and multiparameter secretion characteristics. Such further advances depend upon enhancing capabilities to better match physiologic immune environments. Abilities such as providing precisely defined dynamic inputs, increased use of co-cultures, increased usage of multiparameter readouts, and improved culture environments, when coupled with single-cell level characterizations, will enable investigations more deeply probing secretion.

## Quantitative analysis of cell migration

Immune related migration is involved in a diverse set of functions employed to defend against immune challenges. Migration includes immediate homing of innate immune first responders to infection sites, extravasation of cells leaving circulation and entering a site of inflammation, migration through the thymus during B-cell and T-cell maturation, and migration during wound healing and tissue remodeling, as well as local movements at sites of inflammation. These movements depend upon coordination between different populations of cells, and must change markedly at different time points. Microfluidics has been used extensively in cellular migration research in a diverse set of applications.<sup>55–59</sup> For immune applications, much work has focused on platforms to recreate multiparameter, dynamic aspects of *in vivo* immune migration. Several principle methods have strived to recapitulate relevant signals present in specific scenarios. These systems also have been created to allow high-quality, real time observation of cell responses, a key functionality lacking in more conventional measurement systems.

Microfluidic chips creating constant perfusion of compounds are one important method frequently used to investigate migration. They establish conditions supplying signals such as cytokines and nutrients, often with relatively simple systems. Such a system for measuring cell attachment under flow conditions was used by Kim and Moon *et al.* to investigate factors governing T-cell adhesion.<sup>60</sup> In their work, channel surfaces were coated with T-cell attachment molecules E-selectin, ICAM-1, and VCAM-1 to study adhesion and migration. Different coating levels and combinations were used allowing relative influences upon T-cell adhesion to be measured under flow. This arrangement addressed deficiencies with *in vitro* and *in vivo* systems suffering from reproducibility. This is due to the fact that other systems are not strictly defined in attachment chemistry, making analysis of specific functional components difficult to determine. Perfusion was also used to study neutrophil rolling under flow by Sundd *et al.*<sup>61</sup> Microchannel surfaces were functionalized with different densities of P-selectin to visualize the slings and tethers used to mediate cell rolling. They observed shear dependent sling length and could uncover the mechanics of how slings and tethers allow rolling in high shear environments.

Perfusion systems are also capable of producing gradients within microfluidic chips. Gradients are naturally of great



interest within the context of immunity as they supply cues for cellular positioning, movement and decision making. Flow of gradients over cells was used by Nandagopal and Wu *et al.* for measuring primary T-cell migration in a situation mimicking lymph node related movements.<sup>62</sup> The effects of competing, diffusive based gradients of CCL21 and CCL19 which are principle cytokines governing attraction to and migration within lymphoid tissues were examined. Ultimately migration in the context of these multiple signals was seen to be related to receptor availability. These cytokines share a receptor, and the system allowed for clear observation of cells in areas of different concentrations of the two competing chemokines. Similarly, Kim and Haynes used mixed flow streams to establish competing gradients over primary neutrophils.<sup>63</sup> They tested combinations of the chemokines and chemoattractants, formyl-met-leu-phe, CXCL8, CXCL2, and leukotriene B4. Their measurements encompassed migration speed, direction, and effective migration in relation to different gradients. They detected effects of gradient combinations such as an increase in migration speed when two chemoattractants are present and uncovered distinct hierarchies of the chemoattractants driving cellular motion. Diffusive, flow based gradients were also used by Ricart *et al.* to study both directionality and traction forces of migrating dendritic cells.<sup>64,65</sup> They measured hierarchical relationships between CCL19, CCL21, and CXCL12, pertaining to dendritic cells. Observation of dendritic cell traction force during migration was *via* incorporation of force sensing microposts inside channels.

An improved assay, also using channels to establish gradients, but removing the often detrimental need for flow over cells has recently been developed by our group.<sup>66</sup> This chip functions by temporally modulating the flow (flow-switching) that establishes source and sink reservoirs on either side of an assay chamber seeded with cells. These side chambers are periodically replenished while the assay chamber is closed. The assay chamber is then briefly opened to allow the source and sink to be replenished through molecular diffusion, Fig. 3A. The result is that the cell culture chamber itself experiences diffusion of signaling molecules without cross flow. Establishment and individual programming of 30 independent gradients with multiday stability is achievable on a single chip. This allowed for multiple complex gradient conditions with replicates in a single experiment. While the gradient is for all practical purposes indefinitely stable, it could also be temporally changed by modifying flow supplied to side chambers. This could be easily implemented with automated capabilities of the computer controlled membrane-valve based system. Additionally, with flow over cells eliminated, the assay becomes more physiologically relevant, as secreted molecules are not washed out of the cell chamber. Using this gradient generation method combined with fluorescent reporters and live cell microscopy; real time activation, migration and proliferation of 3T3 fibroblasts and RAW 264.7 macrophages were observed. Interesting behavior such as cell concentration dependent proliferation and migration,

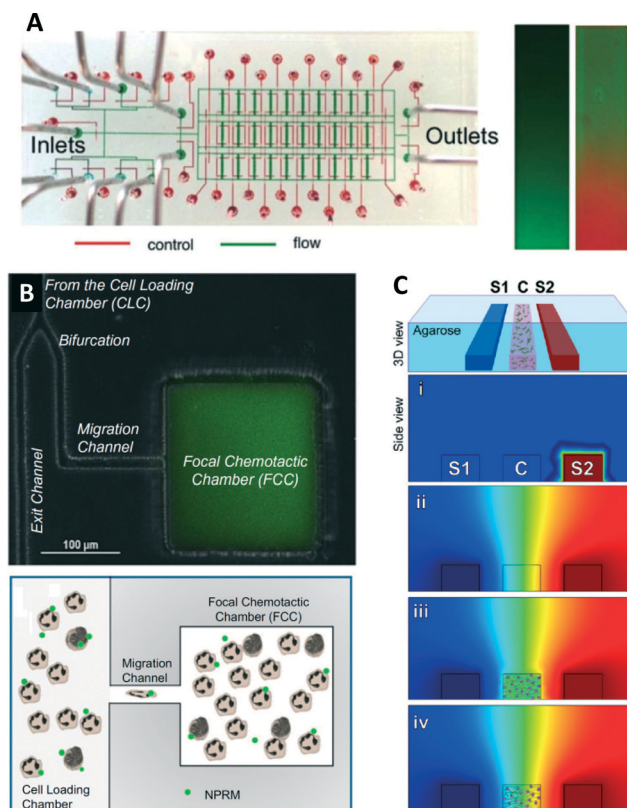


Fig. 3 Measuring immune cell migration. A) Stable, programmable gradient chip. Reproduced from ref. 66. B) Multiassay migration chip<sup>67</sup> C) gel-based gradient migration chip.<sup>78</sup>

and paracrine immune activation was uncovered. In the case of paracrine signaling, it was seen to provoke delayed NF-κB activation of neighboring cells which could not have been observed under other systems relying on flow based gradients or gradients which are not temporally defined.

Another set of methods for gradient formation relies on using channels of a reduced cross section to achieve certain functions. These include flow restriction, gradient establishment, and for other purposes such as mimicking bodily structures. Such a method for passive gradient formation has been developed by Jones *et al.* to recreate a focal inflammation scenario.<sup>67</sup> In addition to migration, the system looked into several other aspects of cells moving towards a source of inflammation. The device incorporated a large central chamber into which cells were seeded, connected *via* lower channels to 16 peripheral chambers primed with chemoattractants, Fig. 3B. Cells seeded in the central chamber experienced a gradient of attractant established from peripheral chambers and migrated towards them over time periods up to 20 hours. Additionally, a fluorescent elastase assay was incorporated into the device. This probed the inflammatory character of induced migration by detecting elastin breakdown by migrating cells. Going even further, the design incorporated a channel bifurcation which would distinguish between cells displaying only increased chemokinesis, as they would divide equally at the bifurcation, and cells migrating



chemotactically, which would turn towards higher gradient values. A combination of primary neutrophils and monocytes under various chemoattractants and under the effect of several immunomodifiers were observed. The benefit of such a diverse system is the output of multiparameter observations of several interesting facets of this immune scenario. This system was able to uncover not only migratory characteristics, but also systems level properties such as the influence of co-culturing monocytes and neutrophils on elastase and migration activities.

A similar small channel gradient method was used by Ambravaneswaran *et al.* to investigate neutrophil migration and decision making.<sup>68</sup> Neutrophils were placed in bifurcated microchannels and their decisions on path length in a gradient context were examined. Directional signal making was also examined with posts obstructing migration inside channels used to provoke splitting of the migrating cell leading edge. This observation was used to postulate that dynamic, tension based instability is used by neutrophils to ultimately retract one cell front and continue migration.

Others have used small channels as a means to mimic vascular connections separating populations of cells. Businaro and De Ninno *et al.* developed such a microfluidic system to investigate immune surveillance of cancer cells.<sup>69</sup> The system consisted of co-culturing a melanoma cell line separated from splenic primary immune cell types. Small connecting channels mimicked vascular connections and allowed observation of extravasation. Time lapse tracking of fluorescent cells for up to one week showed several interesting behaviors of this system. Immune compromised IRF-8 knockout cells, for example, were seen to migrate towards cancer cells to a significantly lesser degree compared to wild type cells. More interestingly, it was seen that knockout cells provoked increased migration of melanoma cells, consequently increasing their invasiveness.

Small channels have been used for several other investigations including neutrophil extravasation through narrow restrictions,<sup>70</sup> migration from burn patient neutrophils,<sup>71</sup> chemokine driven T-cell recruitment,<sup>72</sup> and a simple open system compatible with both robotic automation and standard well plate formats that was used for high throughput neutrophil migration measurements in response to a cytokine gradient.<sup>73</sup>

Others such as Keenan *et al.* used channel restrictions and fluorescent dyes to produce dynamic gradients for tracking migration while simultaneously detecting gradient profiles.<sup>74</sup> A central channel separated two side channels by small connecting channels for study of neutrophil movements. A similar design for gradient formation was demonstrated by Shamloo *et al.* to investigate mast cell migration.<sup>75</sup> They saw interesting single-cell migratory characteristics such as low concentration repulsion and time-dependent chemoattraction to Kit ligand gradients.

Work combining migration with activation was developed by Mitra and Jindal for examining dendritic cell movement with T-cell calcium release.<sup>76</sup> They created a gradient of

CCL19 *via* channel restrictions to induce dendritic cell migration. Once cells had migrated sufficiently, they would become trapped in another area of the chip. This area was subsequently loaded with T-cells from the same donor, and T-cell activation was detected by  $\text{Ca}^{2+}$  fluorescence. Using this method, systems-level differences between activated and non-activated dendritic cells upon T-cells as well as the effect of dendritic cell migration on activation potential could be observed.

Other important microfluidic methods attempt to create systems more closely resembling physiological three-dimensional environments. Such constructs should allow cells to display more realistic behaviors. These methods are typically based upon using gels for creation of 3D structures and encapsulation of cells. Han *et al.* for example, investigated 3D neutrophil extravasation with a system that supplied an inflammatory gradient and an extracellular matrix through which cells could migrate.<sup>77</sup> This system improved visibility and control over migration, and allowed for 3D observation of transendothelial migration.

Lymphoid migration was likewise studied by Haessler *et al.* with a 3D system that measured dendritic cells,<sup>78,79</sup> Fig. 3C. This device consisted of ECM based channels with reservoirs of chemokines CCL21 and CCL19 separated from a central cell channel. They could also physically anchor CCL21 to the matrix mimicking physiologic immobilization. The separation and gel established a gradient and allowed for the 3D based observation that CCL21 exercises a stronger attractive potential *versus* CCL19. This was found, in agreement with the observations by Nandagopal and Wu,<sup>62</sup> to be likely due to receptor recycling differences between the two cytokines.

3D study of the lymph node environment was also conducted in a perfused gel environment by Tomei *et al.*<sup>80</sup> They observed key activities dependent upon growth of lymph node T zone fibroblastic reticular cells inside an optimized 3D environment. These included more physiologic display of both cytokine expression and cell organization. Using this system they postulated that flow through lymph nodes could be a key early stage immune signal used to recruit cells for immune upregulation.

## Cell removal

An indispensable aspect of immune function is removal of invading pathogens and damaged host cells. This occurs in conditions of pathogen elimination, elimination of infected cells, or simply as a normal part of removing worn out cells or tissue remodeling. Fundamental to this function is formation of cell to cell, or cell to organism contact. Microfluidic constructs are useful for achieving this as their microscale dimensions facilitate establishing contact and, as in other areas, allow dynamic observations.

Natural killer (NK) cell mediated target cell lysis and other behaviors have been observed inside a microwell based system created by Guldevall *et al.*,<sup>81</sup> Fig. 4A. Spatial confinement allowed tracking of motile cells over extended time periods

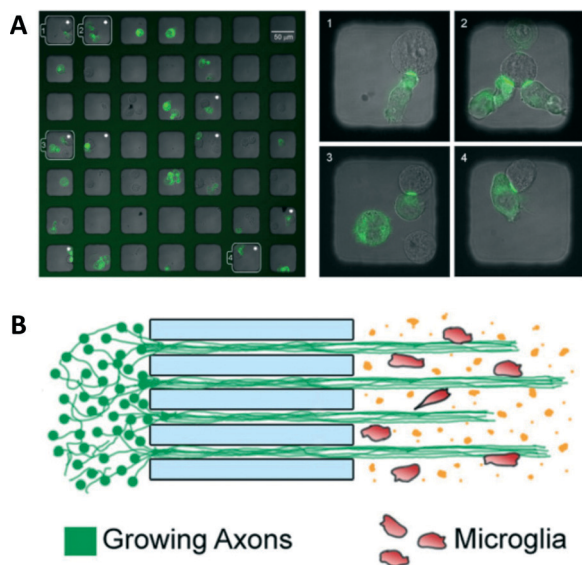


Fig. 4 Monitoring cell removal. A) Microwell based system for NK cells<sup>81</sup> B) system to examine phagocytosis of neurons by microglia (modified with permission from ref. 86).

and facilitated NK cells encountering target cells. The chip consisted of up to 32 400 chambers and their assay involved primary and cell line NK cells lysing target cell lines as well as forming immune synapses. For lysing, they observed two kinds of lytic behavior (slow and fast) by detecting escape of the cytosolic marker calcium green from target cells after NK cell action. Additionally they observed immune synapse formation between NK and target cells *via* localized fluorescence of the synapse marker HLA-Cw6-GFP. The microwell array was initially screened at low magnification and after identifying promising cell pairs, high resolution imaging of synapse formation was conducted on this subset. Further work by this group included study of migration in the context of lysis,<sup>82</sup> additional work relating to heterogeneity of cell elimination,<sup>83</sup> and incorporation of acoustic aggregation to facilitate cell–cell associations.<sup>84</sup>

Additional use of the microwell system described above,<sup>28</sup> was employed by Hassin and Garber *et al.* to examine T-cell lytic behavior.<sup>85</sup> Lytic ability was examined in terms of fast, perforin based, and slow, Fas ligand based elimination of target cells. It could be seen by tracking cells inside microwells that slower Fas-based lytic ability can complement faster but heterogeneous perforin based ability. This was detected *via* correlating off-chip fluorescent staining of lytic components to on-chip monitoring of cell lysis. Overall this behavior was theorized to ensure optimal cell removal by T-cells.

A different type of system using channels was developed by Hosmane and Tegenge *et al.* to study phagocytosis of axons by microglia.<sup>86</sup> This device consisted of separate compartments for neurons and microglia. These were connected by long channels through which axons formation was guided *via* chemical patterning and physical confinement. This provided microglia contact to axons but not to neuron bodies,

and allowed microglial independent stimulations of neurons, Fig. 4B. The combined use of channels and patterning ensured reproducible, and easy measurement of axon formation. Once axons from primary rat hippocampal neurons had extended through channels, primary rat microglia were introduced, and phagocytosis was monitored. Under various conditions of specific inhibition and stimulation, increased or decreased axon retraction could be observed. This led to the conclusion that TRIF and p38 MAPK expression in microglia play key roles in damaged neuron removal.

## Antibody production

Creating antibodies that bind a specific biomolecule is the key function of the adaptive immune response allowing the body to fight off new as well as established immune challenges. Understanding how this process occurs and could be manipulated is a key goal in areas ranging from controlling infections and cancer to treating autoimmune conditions. Additionally, developments in this area are of use beyond understanding immunity such as for drug and assay development, applications for which antibodies have established and expanding roles.

To this end, several developments have occurred implementing on chip study of antibody secreting cells. These have included approaches such as employing beads to measure binding kinetics from antibodies secreted by single hybridomas<sup>87</sup> to other methods designed to analyze large numbers of cells. To overcome the often limiting requirement of conducting high numbers of plate based assays, these high-throughput methods employed single-cell isolation, and subsequent assays to screen for functional antibodies. A method using droplet isolation was employed by Debs *et al.* which assayed cells for not only binding but also functionality of secreted antibodies, Fig. 5A.<sup>88</sup> With their system, a hybridoma cell line was encapsulated in droplets which were later fused with a fluorescent enzymatic substrate for angiotensin converting enzyme 1 (ACE-1). Cells which secreted a functional antibody blocked the enzyme action and rendered their droplet dim compared to those not secreting a functional antibody. This allowed for a 9 400-fold enrichment to be achieved and for subsequent further analysis of selected cells.

Using microwells for isolation, Kinoshita and Ozawa *et al.* seeded cells in a chip possessing up to 45 000 chambers. They then combined two fluorescent detection methods (internal calcium ion and surface receptor labeling) to achieve a population screening sensitivity of 0.05%.<sup>89</sup> Detection of binding induced calcium ion spikes coupled with localized surface binding of tagged antigen reduced false positives by selecting only for double positive events. Other applications by this group employed microwells to directly analyze the functionality of antibodies released by primary human, and other cells,<sup>90,91</sup> Fig. 5B. With human cells, functionalized microwells captured secreted antibodies to screen plasma cells from donors inoculated with influenza and

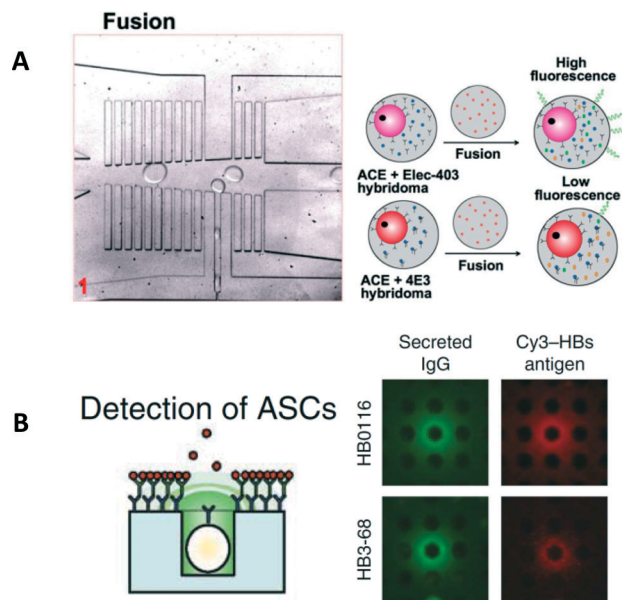


Fig. 5 Antibody interrogation using microfluidic systems. A) Droplet based assaying of antibody functionality<sup>88</sup> B) microwell detection of antibody binding. Adapted by permission from Macmillan Publishers Ltd: *Nat. Med.*,<sup>90</sup> copyright (2009).

hepatitis B proteins. Captured antibodies were assayed for specificity by probing with related fluorescently tagged proteins. Subsequent fluorescent tagging of the antibody and comparison to amount of antigen bound showed not only if cells could produce an antibody against the pathogen, but also antibody affinity.

Park and Han *et al.*<sup>92</sup> also used microwells to capture antibodies during multiple successive screenings. They studied Chinese hamster ovary clones by capturing antibodies on the well lid. The lid was subsequently probed fluorescently to identify competent cells for expansion. Using repeated rounds of selection and screening, they could quickly uncover high producing clones and recover these cells for additional study.

Microwells can also be used to study specific antibody binding abilities as was done by Ogunniyi *et al.*<sup>93</sup> They captured antibodies produced by mouse hybridomas on a slide to generate a protein array. This array of spots was then probed with fluorescently tagged proteins to indicate which cells produced antibodies capable of binding specific proteins. Functional cells could then be correlated to spots and recovered for further analysis.

Additional use of a microwell array was demonstrated by Gong *et al.*<sup>94</sup> They measured antibody related RNA production by in-well RT-PCR to aid in understanding cellular antibody productions. They looked at 6000 hybridoma cells and interestingly saw that only a fraction of cells that transcribed antibody related RNA actually secreted antibodies.

Finally, a microfluidic chromatin immunoprecipitation method was developed by Wu *et al.* using antibody functionalized beads as a capture substrate.<sup>95</sup> The microfluidic nature of the device improved signal strength as well as increased

throughput by means of parallel sample processing. The device was demonstrated for several applications including rapidly screening for ChIP antibodies, and detecting fine temporal resolution of transcription factor and histone binding of DNA introduced into chips.

The microfluidic techniques that have been developed demonstrated several useful features for understanding antibody functions. These included observation of multiple aspects of antibody behavior and production as well as eliminating the need to expand cells, improved assay times, ability to handle large numbers of cells, and ability to screen for specific antigens. The use of microfluidics was key to achieving these advances by facilitating cell handling, and assay operations. Due to these demonstrated abilities and interest in this area, future microfluidic based developments are likely to occur for antibody study and screening applications.

## Drug screening

Identifying the effect of drugs on the immune system is a critical requirement for discovery of disease treatments as well as other applications such as developing useful compounds for experimental perturbation of cells. Microfluidic systems offer possibilities to increase the number of conditions being screened, allow drug induced time-varying perturbations to be monitored, and reduce times and costs to conduct such tests. Probing the complex and dynamic behavior embodied by the immune system with microfluidics is especially relevant to this area, as drug effects are often wide ranging and complex. Crucial systems-level drug effects can be studied allowing for increased knowledge and parameter space and consequently more effective and rapid identification of useful compounds and their optimal administration. Capabilities offered by microfluidics include exposure of cells to a large range of inputs, screening of high numbers of cells and conducting direct, dynamic observations of drug effects. Microfluidics also allow clearer observation of drug-cell interactions especially when single cells are observed as averaging effects and confounding interactions with other bodily elements are eliminated.

A microfluidic approach for RNA interference screening was created by Schudel *et al.* by merging microarrays with microfluidic channels.<sup>96</sup> RNA sequences targeting virus entry to HeLa cells were first spotted on a surface using a removable set of microchannels. After this, a second set of microchannels was aligned to the spots and cells were introduced for transfection and observation. Effectiveness of virus entry inhibition was fluorescently detected from readout of a model virus which fluoresced upon infection. This method demonstrated ability to screen for 8 different compounds with 12 repetitions in a rapid and inexpensive fashion.

A simple, passive microfluidic platform for screening effects of soluble compounds was developed by Berthier and Lim *et al.*<sup>97</sup> Cells were exposed to gradients of compounds established across a reduced cross sectional area connected to source and sink reservoirs. The method identified the



effect of fungal secondary metabolites on primary human neutrophil migration with only minimal pipetting or control.

Frisk *et al.* used a microwell based system for compound screening and culture of cells over time periods of up to several days.<sup>36</sup> Their reusable system consisted of various sized silicon wells with a maximum of 102 400 wells per chip. They demonstrated a B-cell screening assay discriminating between cells able to live in the presence of an antibiotic and those that could not. They also demonstrated a degree of automated image analysis to address working with high cell numbers.

Park *et al.* developed a circulatory model system to analyze effects of inhibiting drugs on attachment of T-cells under physiologically relevant conditions.<sup>98</sup> The system cultured a monolayer of human umbilical vein endothelial cells (HUVEC) inside a microchannel so that a flow of T-cells could interact with them. Such a system allowed the shear dependent adhesion of T-cells to be observed. Various scenarios of HUVEC activation and T-cell inhibiting drugs were combined to understand the system of interactions between cells under conditions of fluid flow. In this way, drug dependent adhesion of Jurkat as well as adhesion of primary T-cells from healthy and Lupus patient sources was studied.

Finally, Brouzes *et al.* developed a high throughput droplet based system that demonstrated screening a drug concentration range and detecting monocyte viability.<sup>99</sup> They combined droplets containing drugs plus a label, with droplets containing cells. Cells were incubated with the compound, and a subsequent fluorescent assay indicated cell death. Specific drugs or concentrations supplied to each cell could be referenced *via* its labeling. This allowed for correlating effects of hundreds of drugs on thousands of cells with the throughput advantages of droplet microfluidics.

## Future outlook and conclusions

An increasing number of microfluidic systems have addressed key challenges in the study of complex immune functions, including single-cell analysis, dynamics and multiparameter measurements in a systems context. Much, however, remains to be uncovered about details of the immune system and how to modify aberrant immune responses to improve healthcare. Advances in the quantification of the overall dynamic cell picture will provide much needed data sets to calibrate computer models that aim at predicting system behavior in complex scenarios such as infection and drug response. This would include combining measurements from proteomics, secretion dynamics, nucleic acid analysis, cell-cell interactions, and functional attributes such as migration, lysis, apoptosis and others. As well as combining types of measurements is the need to increase the number of parameters of each measurement type. Activity of even a single immune cell is highly complex, integrating dozens of molecules and pathways, and regulating hundreds of genes. Thus, typical experiments analyzing one or a few parameters are woefully inadequate for understanding a given immune function. Cellular variability also implies that

multi-parameter measurements should be conducted, to the greatest extent possible, on the same single cells.

Advances are also needed for more basic cell culture system functions. The increased use of primary human cells is one area where this improvement is increasingly feasible. Many animal models are poor substitutes for human biology<sup>100</sup> and increasing regulations make animal testing prohibitive in many contexts. Direct usage of primary human cells sidesteps relevance issues relating to use of cell lines and non-human cells. Co-culture systems can directly observe the interactive nature of many immune functions and are thus necessary tools to study these interactions. Many opportunities exist to improve system environments ensuring they more closely resemble physiological systems. This requires improved chemistry, geometry, and increased control over spatial and temporal signals. Spatially and temporally defined arrangement of cells (*i.e.* immune cells and pathogens), their stimulation and retrieval can advance culture system capabilities closer to *in vivo* conditions, and such approaches are a next frontier for microfluidics research in cell and systems biology. Complementing these advances, opportunity exists for culturing larger numbers of cells as well as tissues, organs, and organoids towards the goal of recreating a complete immune system analog inside a microfluidic device.

The increased scale of data gained from such systems means measurement and analysis abilities must also improve. This entails automation of both data generation and interpretation. Automation of microfluidic systems is to a large degree technically feasible, though not often by simple means. Automation however, when implemented, not only enables control of basic system operations but also provides advanced functionalities such as real-time closed-loop feedback control. Feedback control can improve stability of experimental conditions, and allow driving cell populations and gene expression levels to desired set points with minimal variability.<sup>101</sup> More importantly, cell tracking and analysis presents significant challenges especially when highly motile cells are to be studied. Image analysis becomes limiting when millions of images, easily generated by parallelized microfluidic systems, must be analyzed. Beyond imaging, overall computational analysis of data must improve in terms of speed and quality. This is imperative if the meaning contained in complex data sets is to be translated into useful form.

Further advances using microfluidics to understand immunity rely on extending and using capabilities to more effectively deal with challenges presented by systems immunology. Developing technologies first, and searching for applications later, an approach often seen in the engineering community, is likely to fail to meet the challenges presented by urgent problems in systems immunology. Technologies originating from already established problems in biology and medicine are well positioned to make an impact. This requires experts in different fields, *e.g.* engineers and biologists to understand each other's language and have the necessary scientific literacy in multiple disciplines. Successful

collaborations based on efficient communication will lead to developing microfluidic systems performing dynamic, multiparameter single-cell measurements immediately relevant to open and urgent immune related problems. This will lead to an increasing amount of immune data, higher data quality and eventually predictive *in silico* models of the immune system. This can ultimately lead to treatments for conditions that have eluded traditional analysis.

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## References

- W. C. Koff, D. R. Burton, P. R. Johnson, B. D. Walker, C. R. King, G. J. Nabel, R. Ahmed, M. K. Bhan and S. A. Plotkin, *Science*, 2013, **340**, 1232910.
- J. J. Hornberg, F. J. Bruggeman, H. V. Westerhoff and J. Lankelma, *BioSystems*, 2006, **83**, 81–90.
- D. Young, J. Stark and D. Kirschner, *Nat. Rev. Microbiol.*, 2008, **6**, 520–528.
- A. Pujol, R. Mosca, J. Farrés and P. Aloy, *Trends Pharmacol. Sci.*, 2010, **31**, 115–123.
- A. Aderem, J. N. Adkins, C. Ansong, J. Galagan, S. Kaiser, M. J. Korth, G. L. Law, J. E. McDermott, S. Proll, C. Rosenberger, G. Schoolnik and M. G. Katze, *mBio*, 2011, **2**, e00325–00310.
- M. Mehling and S. Tay, *Curr. Opin. Biotechnol.*, 2014, **25**, 95–102.
- B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter, *Mol. Biol. Cell*, Garland science, Taylor & Francis Group, 2007.
- A. Eldar and M. B. Elowitz, *Nature*, 2010, **467**, 167–173.
- S. Tay, J. J. Hughey, T. K. Lee, T. Lipniacki, S. R. Quake and M. W. Covert, *Nature*, 2010, **466**, 267–271.
- G. Lahav, N. Rosenfeld, A. Sigal, N. Geva-Zatorsky, A. J. Levine, M. B. Elowitz and U. Alon, *Nat. Genet.*, 2004, **36**, 147–150.
- M. B. Elowitz, A. J. Levine, E. D. Siggia and P. S. Swain, *Science*, 2002, **297**, 1183–1186.
- B. Snijder, R. Sacher, P. Ramo, E.-M. Damm, P. Liberali and L. Pelkmans, *Nature*, 2009, **461**, 520–523.
- A. A. Cohen, N. Geva-Zatorsky, E. Eden, M. Frenkel-Morgenstern, I. Issaeva, A. Sigal, R. Milo, C. Cohen-Saidon, Y. Liron, Z. Kam, L. Cohen, T. Danon, N. Perzov and U. Alon, *Science*, 2008, **322**, 1511–1516.
- R. Gómez-Sjöberg, A. A. Leyrat, D. M. Pirone, C. S. Chen and S. R. Quake, *Anal. Chem.*, 2007, **79**, 8557–8563.
- Y. Awwad, T. Geng, A. S. Baldwin and C. Lu, *Anal. Chem.*, 2012, **84**, 1224–1228.
- I. Zaretsky, M. Polonsky, E. Shifrut, S. Reich-Zeliger, Y. Antebi, G. Aidelberg, N. Waysbort and N. Friedman, *Lab Chip*, 2012, **12**, 5007–5015.
- R. Cheong, C. J. Wang and A. Levchenko, *Mol. Cell. Proteomics*, 2009, **8**, 433–442.
- N. Srivastava, J. S. Brennan, R. F. Renzi, M. Wu, S. S. Branda, A. K. Singh and A. E. Herr, *Anal. Chem.*, 2009, **81**, 3261–3269.
- M. Wu, T. D. Perroud, N. Srivastava, C. S. Branda, K. L. Sale, B. D. Carson, K. D. Patel, S. S. Branda and A. K. Singh, *Lab Chip*, 2012, **12**, 2823–2831.
- D. R. Gossett, H. T. K. Tse, S. A. Lee, Y. Ying, A. G. Lindgren, O. O. Yang, J. Rao, A. T. Clark and D. Di Carlo, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 7630–7635.
- D. E. Clapham, *Cell*, 2007, **131**, 1047–1058.
- M. Kirschbaum, M. S. Jaeger and C. Duschl, *Lab Chip*, 2009, **9**, 3517–3525.
- X. R. Bao, I. D. C. Fraser, E. A. Wall, S. R. Quake and M. I. Simon, *Biophys. J.*, 2010, **99**, 2414–2422.
- S. L. Faley, M. Copland, D. Wlodkowic, W. Kolch, K. T. Seale, J. P. Wikswo and J. M. Cooper, *Lab Chip*, 2009, **9**, 2659–2664.
- K. R. Duffy, C. J. Wellard, J. F. Markham, J. H. S. Zhou, R. Holmberg, E. D. Hawkins, J. Hasbold, M. R. Dowling and P. D. Hodgkin, *Science*, 2012, **335**, 338–341.
- N. Zurgil, E. Afrimzon, A. Deutsch, Y. Namer, Y. Shafran, M. Sobolev, Y. Tauber, O. Ravid-Hermesh and M. Deutsch, *Biomaterials*, 2010, **31**, 5022–5029.
- D. Day, K. Pham, M. J. Ludford-Menting, J. Oliaro, D. Izon, S. M. Russell and M. Gu, *Immunol. Cell Biol.*, 2008, **87**, 154–158.
- Y. S. Schiftenbauer, Y. Kalma, E. Trubniykov, O. Gal-Garber, L. Weisz, A. Halamish, M. Sister and G. Berke, *Lab Chip*, 2009, **9**, 2965–2972.
- N. Dénervaud, J. Becker, R. Delgado-Gonzalo, P. Damay, A. S. Rajkumar, M. Unser, D. Shore, F. Naef and S. J. Maerkl, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 15842–15847.
- R. J. Taylor, D. Falconnet, A. Niemistö, S. A. Ramsey, S. Prinz, I. Shmulevich, T. Galitski and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 3758–3763.
- A. R. Wu, N. F. Neff, T. Kalisky, P. Dalerba, B. Treutlein, M. E. Rothenberg, F. M. Mburu, G. L. Mantalas, S. Sim, M. F. Clarke and S. R. Quake, *Nat. Methods*, 2014, **11**, 41–46.
- R. Zenobi, *Science*, 2013, **342**, 1243259.
- Y. S. Shin, F. Remacle, R. Fan, K. Hwang, W. Wei, H. Ahmad, R. D. Levine and J. R. Heath, *Biophys. J.*, 2011, **100**, 2378–2386.
- C. Ma, R. Fan, H. Ahmad, Q. Shi, B. Comin-Anduix, T. Chodon, R. C. Koya, C.-C. Liu, G. A. Kwong, C. G. Radu, A. Ribas and J. R. Heath, *Nat. Med.*, 2011, **17**, 738–743.
- Y. Lu, J. J. Chen, L. Mu, Q. Xue, Y. Wu, P.-H. Wu, J. Li, A. O. Vortmeyer, K. Miller-Jensen, D. Wirtz and R. Fan, *Anal. Chem.*, 2013, **85**, 2548–2556.
- T. Frisk, M. Khorshidi, K. Guldevall, B. Vanherberghen and B. Önfelt, *Biomed. Microdevices*, 2011, **13**, 683–693.
- Q. Han, N. Bagheri, E. M. Bradshaw, D. A. Hafner, D. A. Lauffenburger and J. C. Love, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 1607–1612.
- Y. J. Yamanaka, G. L. Szeto, T. M. Gierahn, T. L. Forcier, K. F. Benedict, M. S. N. Brefo, D. A. Lauffenburger, D. J. Irvine and J. C. Love, *Anal. Chem.*, 2012, **84**, 10531–10536.

- 39 Q. Song, Q. Han, E. M. Bradshaw, S. C. Kent, K. Raddassi, B. r. Nilsson, G. T. Nepom, D. A. Hafler and J. C. Love, *Anal. Chem.*, 2009, **82**, 473–477.
- 40 A. J. Torres, R. L. Contento, S. Gordo, K. W. Wucherpfennig and J. C. Love, *Lab Chip*, 2013, **13**, 90–99.
- 41 N. Varadarajan, B. Julg, Y. J. Yamanaka, H. Chen, A. O. Ogunniyi, E. McAndrew, L. C. Porter, A. Piechocka-Trocha, B. J. Hill, D. C. Douek, F. Pereyra, B. D. Walker and J. C. Love, *J. Clin. Invest.*, 2011, **121**, 4322–4331.
- 42 Y. J. Yamanaka, C. T. Berger, M. Sips, P. C. Cheney, G. Alter and J. C. Love, *Integr. Biol.*, 2012, **4**, 1175–1184.
- 43 N. Varadarajan, D. S. Kwon, K. M. Law, A. O. Ogunniyi, M. N. Anahtar, J. M. Richter, B. D. Walker and J. C. Love, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 3885–3890.
- 44 T. Konry, M. Dominguez-Villar, C. Baecher-Allan, D. A. Hafler and M. L. Yarmush, *Biosens. Bioelectron.*, 2011, **26**, 2707–2710.
- 45 V. Chokkalingam, J. Tel, F. Wimmers, X. Liu, S. Semenov, J. Thiele, C. G. Figdor and W. T. S. Huck, *Lab Chip*, 2013, **13**, 4740–4744.
- 46 N.-T. Huang, W. Chen, B.-R. Oh, T. T. Cornell, T. P. Shanley, J. Fu and K. Kurabayashi, *Lab Chip*, 2012, **12**, 4093–4101.
- 47 W. Chen, N.-T. Huang, B. Oh, R. H. W. Lam, R. Fan, T. T. Cornell, T. P. Shanley, K. Kurabayashi and J. Fu, *Adv. Healthcare Mater.*, 2013, **2**, 921–921.
- 48 M. Bielefeld-Sevigny, *Assay Drug Dev. Technol.*, 2009, **7**, 90–92.
- 49 H. Zhu, G. Stybayeva, J. Silangcruz, J. Yan, E. Ramanculov, S. Dandekar, M. D. George and A. Revzin, *Anal. Chem.*, 2009, **81**, 8150–8156.
- 50 Y. Liu, J. Yan, M. C. Howland, T. Kwa and A. Revzin, *Anal. Chem.*, 2011, **83**, 8286–8292.
- 51 Y. Liu, T. Kwa and A. Revzin, *Biomaterials*, 2012, **33**, 7347–7355.
- 52 C. F. Nathan, *J. Clin. Invest.*, 1987, **79**, 319–326.
- 53 J. Yan, V. A. Pedrosa, J. Enomoto, A. L. Simonian and A. Revzin, *Biomicrofluidics*, 2011, **5**, 032008–032011.
- 54 Y. Shafran, N. Zurgil, E. Afrimzon, Y. Tauber, M. Sobolev, A. Shainberg and M. Deutsch, *Anal. Chem.*, 2012, **84**, 7315–7322.
- 55 S. Kim, H. J. Kim and N. L. Jeon, *Integr. Biol.*, 2010, **2**, 584–603.
- 56 S. Vedel, S. Tay, D. M. Johnston, H. Bruus and S. R. Quake, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 129–134.
- 57 F.-Q. Nie, M. Yamada, J. Kobayashi, M. Yamato, A. Kikuchi and T. Okano, *Biomaterials*, 2007, **28**, 4017–4022.
- 58 S.-Y. Cheng, S. Heilman, M. Wasserman, S. Archer, M. L. Shuler and M. Wu, *Lab Chip*, 2007, **7**, 763–769.
- 59 Y. Z. Meng, Y. X. Li, C. D. Galvani, G. X. Hao, J. N. Turner, T. J. Burr and H. C. Hoch, *J. Bacteriol.*, 2005, **187**, 5560–5567.
- 60 S. K. Kim, W. K. Moon, J. Y. Park and H. Jung, *Analyst*, 2012, **137**, 4062–4068.
- 61 P. Sundd, E. Gutierrez, E. K. Koltsova, Y. Kuwano, S. Fukuda, M. K. Pospieszalska, A. Groisman and K. Ley, *Nature*, 2012, **488**, 399–403.
- 62 S. Nandagopal, D. Wu and F. Lin, *PLoS One*, 2011, **6**, e18183.
- 63 D. Kim and C. L. Haynes, *Anal. Chem.*, 2012, **84**, 6070–6078.
- 64 B. G. Ricart, B. John, D. Lee, C. A. Hunter and D. A. Hammer, *J. Immunol.*, 2011, **186**, 53–61.
- 65 B. G. Ricart, M. T. Yang, C. A. Hunter, C. S. Chen and D. A. Hammer, *Biophys. J.*, 2011, **101**, 2620–2628.
- 66 T. Frank and S. Tay, *Lab Chip*, 2013, **13**, 1273–1281.
- 67 C. N. Jones, J. Dalli, L. Dimisko, E. Wong, C. N. Serhan and D. Irimia, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 20560–20565.
- 68 V. Ambraveswaran, I. Y. Wong, A. J. Aranyosi, M. Toner and D. Irimia, *Integr. Biol.*, 2010, **2**, 639–647.
- 69 L. Businaro, A. De Ninno, G. Schiavoni, V. Lucarini, G. Ciasca, A. Gerardino, F. Belardelli, L. Gabriele and F. Mattei, *Lab Chip*, 2013, **13**, 229–239.
- 70 A. C. Rowat, D. E. Jaalouk, M. Zwerger, W. L. Ung, I. A. Eydelnant, D. E. Olins, A. L. Olins, H. Herrmann, D. A. Weitz and J. Lammerding, *J. Biol. Chem.*, 2013, **288**, 8610–8618.
- 71 K. L. Butler, V. Ambraveswaran, N. Agrawal, M. Bilodeau, M. Toner, R. G. Tompkins, S. Fagan and D. Irimia, *PLoS One*, 2010, **5**, e11921.
- 72 A. Hoerning, K. Koss, D. Datta, L. Boneschansker, C. N. Jones, I. Y. Wong, D. Irimia, K. Calzadilla, F. Benitez, P. F. Hoyer, W. E. Harmon and D. M. Briscoe, *Eur. J. Immunol.*, 2011, **41**, 2291–2302.
- 73 I. Meyvantsson, E. Vu, C. Lamers, D. Echeverria, T. Worzella, V. Echeverria, A. Skoien and S. Hayes, *J. Immunol. Methods*, 2011, **374**, 70–77.
- 74 T. M. Keenan, C. W. Frevert, A. Wu, V. Wong and A. Folch, *Lab Chip*, 2010, **10**, 116–122.
- 75 A. Shamloo, M. Manchandia, M. Ferreira, M. Mani, C. Nguyen, T. Jahn, K. Weinberg and S. Heilshorn, *Integr. Biol.*, 2013, **5**, 1076–1085.
- 76 B. Mitra, R. Jindal, S. Lee, D. X. Dong, L. Li, N. Sharma, T. Maguire, R. Schloss and M. L. Yarmush, *RSC Adv.*, 2013, **3**, 16002–16010.
- 77 S. Han, J.-J. Yan, Y. Shin, J. J. Jeon, J. Won, H. Eun Jeong, R. D. Kamm, Y.-J. Kim and S. Chung, *Lab Chip*, 2012, **12**, 3861–3865.
- 78 U. Haessler, M. Pisano, M. Wu and M. A. Swartz, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 5614–5619.
- 79 U. Haessler, Y. Kalinin, M. Swartz and M. Wu, *Biomed. Microdevices*, 2009, **11**, 827–835.
- 80 A. A. Tomei, S. Siegert, M. R. Britschgi, S. A. Luther and M. A. Swartz, *J. Immunol.*, 2009, **183**, 4273–4283.
- 81 K. Guldevall, B. Vanherberghen, T. Frisk, J. Hurtig, A. E. Christakou, O. Manneberg, S. Lindström, H. Andersson-Svahn, M. Wiklund and B. Önfelt, *PLoS One*, 2010, **5**, e15453.
- 82 M. A. Khorshidi, B. Vanherberghen, J. M. Kowalewski, K. R. Garrod, S. Lindstrom, H. Andersson-Svahn, H. Brismar, M. D. Cahalan and B. Önfelt, *Integr. Biol.*, 2011, **3**, 770–778.
- 83 B. Vanherberghen, P. E. Olofsson, E. Forslund, M. Sternberg-Simon, M. A. Khorshidi, S. Pacouret,



- K. Guldevall, M. Enqvist, K.-J. Malmberg, R. Mehr and B. Önfelt, *Blood*, 2013, **121**, 1326–1334.
- 84 A. E. Christakou, M. Ohlin, B. Vanherberghen, M. A. Khorshidi, N. Kadri, T. Frisk, M. Wiklund and B. Önfelt, *Integr. Biol.*, 2013, **5**, 712–719.
- 85 D. Hassin, O. G. Garber, A. Meiraz, Y. S. Schifffenbauer and G. Berke, *Immunology*, 2011, **133**, 190–196.
- 86 S. Hosmane, M. A. Tegenge, L. Rajbhandari, P. Uapinyoying, N. G. Kumar, N. Thakor and A. Venkatesan, *J. Neurosci.*, 2012, **32**, 7745–7757.
- 87 A. Singhal, C. A. Haynes and C. L. Hansen, *Anal. Chem.*, 2010, **82**, 8671–8679.
- 88 B. E. Debs, R. Utharala, I. V. Balyasnikova, A. D. Griffiths and C. A. Merten, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 11570–11575.
- 89 K. Kinoshita, T. Ozawa, K. Tajiri, S. Kadowaki, H. Kishi and A. Muraguchi, *Cytometry, Part A*, 2009, **75**, 682–687.
- 90 A. Jin, T. Ozawa, K. Tajiri, T. Obata, S. Kondo, K. Kinoshita, S. Kadowaki, K. Takahashi, T. Sugiyama, H. Kishi and A. Muraguchi, *Nat. Med.*, 2009, **15**, 1088–1092.
- 91 T. Ozawa, X. Piao, E. Kobayashi, Y. Zhou, H. Sakurai, T. Andoh, A. Jin, H. Kishi and A. Muraguchi, *PLoS One*, 2012, **7**, e52383.
- 92 S. Park, J. Han, W. Kim, G. M. Lee and H.-S. Kim, *J. Biotechnol.*, 2011, **156**, 197–202.
- 93 A. O. Ogunniyi, C. M. Story, E. Papa, E. Guillen and J. C. Love, *Nat. Protoc.*, 2009, **4**, 767–782.
- 94 Y. Gong, A. O. Ogunniyi and J. C. Love, *Lab Chip*, 2010, **10**, 2334–2337.
- 95 A. R. Wu, T. L. A. Kawahara, N. A. Rapicavoli, J. V. Riggelen, E. H. Shroff, L. Xu, D. W. Felsher, H. Y. Chang and S. R. Quake, *Lab Chip*, 2012, **12**, 2190–2198.
- 96 B. R. Schudel, B. Harmon, V. V. Abhyankar, B. W. Pruitt, O. A. Negrete and A. K. Singh, *Lab Chip*, 2013, **13**, 811–817.
- 97 E. Berthier, F. Y. Lim, Q. Deng, C.-J. Guo, D. P. Kontoyiannis, C. C. C. Wang, J. Rindy, D. J. Beebe, A. Huttenlocher and N. P. Keller, *PLoS Pathog.*, 2013, **9**, e1003289.
- 98 J. Y. Park, H. O. Kim, K.-D. Kim, S. K. Kim, S. K. Lee and H. Jung, *Analyst*, 2011, **136**, 2831–2836.
- 99 E. Brouzes, M. Medkova, N. Savenelli, D. Marran, M. Twardowski, J. B. Hutchison, J. M. Rothberg, D. R. Link, N. Perrimon and M. L. Samuels, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 14195–14200.
- 100 J. Seok, H. S. Warren, A. G. Cuenca, M. N. Mindrinos, H. V. Baker, W. Xu, D. R. Richards, G. P. McDonald-Smith, H. Gao, L. Hennessy, C. C. Finnerty, C. M. López, S. Honari, E. E. Moore, J. P. Minei, J. Cuschieri, P. E. Bankey, J. L. Johnson, J. Sperry, A. B. Nathens, T. R. Billiar, M. A. West, M. G. Jeschke, M. B. Klein, R. L. Gamelli, N. S. Gibran, B. H. Brownstein, C. Miller-Graziano, S. E. Calvano, P. H. Mason, J. P. Cobb, L. G. Rahme, S. F. Lowry, R. V. Maier, L. L. Moldawer, D. N. Herndon, R. W. Davis, W. Xiao and R. G. Tompkins, *Inflammation and Host Response to Injury*, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 3507–3512.
- 101 A. Miliadis-Argeitis, S. Summers, J. Stewart-Ornstein, I. Zuleta, D. Pincus, H. El-Samad, M. Khammash and J. Lygeros, *Nat. Biotechnol.*, 2011, **29**, 1114–1116.