

## **Clonal expansion under the microscope: Studying lymphocyte activation and differentiation using live cell imaging**

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### **Abstract**

Clonal expansion of lymphocytes is a hallmark of vertebrate adaptive immunity. A small number of precursor cells that recognize a specific antigen proliferate into expanded clones, differentiate and acquire various effector and memory phenotypes which promote effective immune responses. Recent studies establish a large degree of heterogeneity in the level of expansion and in cell state between and within expanding clones. Studying these processes *in vivo*, while providing insightful information on the level of heterogeneity, is challenging due to the complex microenvironment and the inability to continuously track individual cells over extended periods of time. Live cell imaging of *ex vivo* cultures within micro fabricated arrays provides an attractive methodology for studying clonal expansion. These experiments facilitate continuous acquisition of a large number of parameters on cell number, proliferation, death and differentiation state, with single cell resolution on thousands of expanding clones that grow within controlled environments. Such data can reveal stochastic and instructive mechanisms that contribute to observed heterogeneity and elucidate the sequential order of differentiation events. Intercellular interactions can also be studied within these arrays by following responses of a controlled number of interacting cells, all trapped within the same microwell. Here, we describe implementations of live cell imaging within microwell arrays for studies of lymphocyte clonal expansion, portray insights already gained from these experiments, and outline directions for future research. These tools, together with *in vivo* experiments tracking single cell responses, will expand our understanding of adaptive immunity and the ways by which it can be manipulated.

## **Introduction:**

Upon recognition of a cognate antigen, lymphocytes massively proliferate and generate expanded clones of required antigen reactivity, which establish an adequate specific immune response<sup>1</sup>. Clonal expansion is coupled with differentiation of naïve lymphocytes into mature effector and memory cells. These processes are affected by external cues from the microenvironment as well as by autocrine and paracrine signaling from the activated lymphocytes themselves. Consequently, cells interact with each other during clonal expansion, leading to collective phenomena such as clonal competition, clonal dominance, cooperation and by-stander activation<sup>2-7</sup>. These phenomena have important implications in immune pathologies, immunotherapies and vaccine design.

Quantitative measurements of the rates of clonal expansion and the related rates of cell proliferation, cell death and differentiation, are central for modeling immune responses. Until recently, quantitative measurements were performed by following the average response of a number of responding clones, as tracking single clones *in vivo* is technically challenging (Figure 1, left). While providing useful information, important details regarding the variation of response between and within expanding clones are masked by population averaging.

## **Clonal expansion: a heterogeneous process**

In recent years, studying clonal expansion and differentiation of individual cells *in vivo* was made possible, by the use of cell barcoding<sup>8</sup> or by adoptive transfer of small cell numbers that can be individually distinguished by surface marker combinations<sup>9,10</sup>. These studies demonstrated the diversity in the response of a starting population of naïve T cells, all having the same T cell receptor (TCR) and responding to the same antigen (Figure 1, middle). Of note, these experiments revealed striking differences in resulting clone size (also referred to as “family size” or “burst size”), which can range over a few orders of magnitude. Cells from a seemingly homogenous starting population follow a highly heterogeneous response to antigen stimulation. Some cells vigorously expand into very large clones of thousands of cells, while others, despite antigen stimulation as

evidenced by changes in surface marker expression, expanded to a much lesser extent. Clones differed not only by their level of expansion, but also in the resulting patterns of cell differentiation, as substantial interclonal and intracлонаl heterogeneity in cell fate were observed (Figure 1, middle). Intracлонаl heterogeneity was observed also in differentiation of human CD4<sup>+</sup> memory T cells in response to a number of pathogens<sup>11</sup>.

In vivo tracking of individual clones further showed that clonal expansion and T cell differentiation are coupled, as evidenced by correlations between expression of differentiation markers and clone size<sup>8-10,12-15</sup>. In addition, it was demonstrated that expression of proteins associated with T cell differentiation is dependent on the number of cell divisions that cells undergo<sup>16</sup>.

The ability to trace the fate of individual cells revealed the complexity of lymphocyte expansion, and also allowed the construction of mathematical models which aim to explain different aspects of the process. For example, single-cell in vivo data was used to constrain different models tracing cell fate during the formation of effector and memory cells<sup>9</sup>. The only model which was able to fit the experimental data was one in which naïve cells develop first into memory precursors, which can further develop into effector cells.

A number of mechanisms have been suggested to contribute to heterogeneity in T cell diversification, including strength of TCR signaling<sup>10</sup>, intrinsic stochastic variability between cells<sup>17</sup>, extrinsic instruction by environmental signaling<sup>18</sup> and asymmetric cell division<sup>12,19-21</sup>. Interestingly, it has been shown that heterogeneity in clone size and phenotype occurs also in ex-vivo cultures of primary lymphocytes<sup>13,22,23</sup>, even when cells are stimulated in the absence of antigen presenting cells. This suggests that variability in the response of individual cells can be achieved in a minimal system that contains only stimulated T cells, not affected by varying external cues. Heterogeneous responses can thus result from mechanisms that are cell intrinsic and might also be supported by cell-to-cell interactions between the activated T cells, for example by direct contacts within T cell clusters<sup>24-26</sup>, and through interactions by secreted cytokines<sup>27,28</sup>. As the expression of most secreted cytokines and their receptors is controlled by extracellular feedback loops,

intercellular interactions between the expanding lymphocytes themselves affect the level of heterogeneity<sup>29</sup>, in addition to any cell autonomous stochasticity.

To further investigate heterogeneity of clonal expansion, its implications and the underlying mechanisms that generate diversification, it is beneficial to follow expanding lymphocytes dynamically and with a single cell resolution. Such experiments can provide continuous measurements of cell proliferation, death and differentiation events, quantify the rates and the correlations between these processes, and evaluate their dynamic changes during the course of the response (Figure 1, Right).

An experimental system that will enable in depth study of these processes should fulfill three major requirements: A) Enable continuous monitoring of individual cells over the activation process. This will facilitate the observation and quantification of intrinsic variability between the activated cells. B) Enable control over intercellular interactions, to facilitate distinction between cell intrinsic and collective processes. C) Allow a tightly controlled environment. This will facilitate the distinction between cell intrinsic heterogeneity and diversity caused by environmental cues. These requirements can be met by the use of ex-vivo live cell imaging of single lymphocytes during activation and differentiation. Using this method, cells are kept in a tightly controlled environment and are continuously monitored throughout several days. This enables direct observation of division and death events, building and characterizing “family trees” of individual clones, as well as dynamic quantification of gene expression in single cells, to study cell differentiation. Data from such experiments can shed light on the complexity of clonal expansion and differentiation, and allow for the extraction of quantitative parameters (such as division times and gene expression levels) that can be used in the construction of mathematical models describing these processes. Quantitative models can be constrained based on single cell microscopy data, and then reliably predict cell population behavior and statistical properties of expanding clones. In the following, we describe the type of experiments that can be conducted using live cell imaging and, through several examples, elaborate on the types of data that are generated using these experimental systems, and their relevance to the investigation of clonal expansion.

### **Using micro-well arrays for live imaging of single cells**

Long term monitoring of lymphocytes during their activation and differentiation has been enabled in recent years by microwell arrays (MWA)<sup>22,23,30-33</sup>, in which non-adherent and motile cells can be cultured and imaged with single-cell resolution over a number of days. Such arrays are available commercially<sup>23,33</sup>, and can also be fabricated in house using PDMS molds<sup>22,30</sup> or hydrogel<sup>32,34</sup>. These arrays are combined with a standard culture plate<sup>22,32</sup> or a microfluidic chip<sup>35</sup>, in which T or B cells can be trapped with minimal perturbation, receive activation signals and differentiate over the course of several days (Figure 2A). These arrays are compatible with fluorescence microscopy, allowing for monitoring of cell proliferation, death and gene expression<sup>22,33</sup> (Figure 2).

In a typical experiment, lymphocytes are loaded into arrays that contain thousands of microwells, of typical size between 20-100 $\mu$ m. Cells can be activated within the microwells, and other signals can be provided through the culture medium, or by coating of the microwell surface with signaling molecules and ligands<sup>36</sup>. By following only microwells that started with one cell, it is possible to track the progression of clonal expansion, as each clone is confined in one microwell throughout the experiment (Figure 2B). Live cell imaging of the array provides a wealth of data on cellular responses over time, with single cell resolution and adequate statistics on hundreds of expanding clones in parallel (Figure 2). MWAs also allow for investigation of intercellular interactions. Cells confined to the same microwell can interact by physical contact as well as by secreted cytokines, while cells in neighboring microwells can communicate through the shared culture medium, but not through direct cell-cell contact.

### **Quantifying Clonal expansion under the microscope**

Using microwell arrays, proliferation can be directly observed from time-lapse microscopy movies and the division times and cell death events in a cell progeny can be determined. Due to the fact that T and B cells are highly motile and change shape upon activation, tracking of the cells by automated image analysis is extremely challenging. Therefore, division times are usually determined semi-automatically, using a combination of automated image analysis and manual correction<sup>22,23,33,37,38</sup>. Death times can be obtained automatically by including low concentrations of propidium iodide (PI) in the cell growth medium; thus, background is minimal while dead cells are brightly stained<sup>22</sup>.

Construction of clonal “family trees” with respective division times of individual cells has led to new insights regarding the factors that determine lymphocyte fate. Hodgkin and colleagues have coined the term Division Destiny to denote the number of divisions a lymphocyte undergoes before becoming quiescent<sup>23,39</sup>. In these experiments, most cells in a clone experienced a typical number of divisions, which was shown to relate to the size of the founder cell prior to its first division<sup>19</sup>. Heterogeneity in the response stems from differences in the number and timing of divisions between clones in the same experiment. As clonal expansion is exponential, even small differences in proliferation rates and total number of divisions between clones translate into very large differences in the resulting clone size, as experimentally observed.

Another striking observation is the strong correlation between the division times of sibling cells in both T and B cell populations (**Figure 2L**)<sup>22,23,33</sup>. Correlation in death times of sibling cells has also been observed<sup>19</sup>. These findings suggest that there are intrinsic factors which are passed from the founder cell to its daughters at each division and that these factors play a crucial role in determining the length of the cell cycle as well as the number of divisions each cell will undergo<sup>23,33,40</sup>. Recently, imaging of single cells from Fucci cell cycle reporter mice<sup>41</sup> was used to study cell cycle progression of activated lymphocytes<sup>42,43</sup>. Cells from these mice appear red at the G1 phase and turn green at the S/G2/M phase, which enables direct quantification of the duration of the different stages in individual cells<sup>40</sup>. Dowling et al.<sup>42</sup> used single cell analysis to show that the cell cycle of activated B cells is highly variable between cells, and found a strong correlation in the timing of G1 between siblings, suggesting that the inheritable factors which are passed to daughter cells could be linked to cell cycle control.

The live cell imaging studies discussed above demonstrate the importance of cell intrinsic mechanisms to the observed heterogeneity in clonal responses. These findings suggest that the behavior at the population level could be modeled using simple stochastic rules governing the behavior of single cells. This paradigm is the basis of the Cyton model<sup>39,44,45</sup>, which assumes that the fate of a cell is dictated by competing stochastic processes. The good agreement between results of the Cyton model and experimental live cell imaging data implies that stochastic and cell autonomous processes can play major roles in driving clonal heterogeneity.

## **Dynamic gene expression in single cells**

Time-lapse imaging of single cell cultures also allows continuous quantification of protein expression over the course of differentiation. Thus, cell phenotype can be dynamically tracked in parallel with proliferation and cell death. This can be achieved in several ways: A) Using transgenic mice strains expressing fluorescent proteins as reporters for genes of interest<sup>46</sup>. B) Surface proteins, such as cytokine receptors or differentiation markers, can be monitored by dynamic staining: low levels of a fluorescently labeled antibody are added to the cell growth medium, contributing negligible fluorescent background. When a target protein is expressed, the antibody is concentrated at the cell surface, and the level of expression can be measured with a good signal to background ratio (Figure 2B)<sup>22,33</sup>. C) Sequential enzyme-linked immunosorbent assay (ELISA) measurements of secreted proteins of interest. These measurements are performed on the micro-well array at different time points while cells are also monitored using time lapse microscopy within the arrays<sup>35,47</sup>.

This data, in conjunction with data on division times, can be used in order to link proliferation to the expression of genes of interest. This was demonstrated in a study in which times of B cell class switching and development into plasmablasts were measured in single cells, with a combination of a genetically encoded reporter and dynamic staining. Data on times of cell division and differentiation showed a good fit to the cell autonomous Cyton model discussed above, implying that the observed diversity of B cell fates arises from cell intrinsic competing stochastic processes<sup>33</sup>. In another example, live cell imaging of cell-cycle reporters was used to complement in vivo experiments for studying T cell memory formation. These experiments showed that the proliferative ability of CD8<sup>+</sup> T cells is linked to their differentiation into memory or effector fate, and that memory formation results from a transition of a subpopulation of fast cycling cells into a slow cycling state, which is heritable within cell progenies. These findings imply that cells first go through a fast proliferating effector phase before a portion of clones adopt a slow cycling memory phenotype<sup>43</sup>. Other models for CD8<sup>+</sup> T cell memory formation, based on single cell in vivo data and mathematical modeling, suggest that cells first become memory precursors before adopting an effector phenotype<sup>9</sup>, or diverge through early asymmetric segregation of IL2 receptors between sibling cells<sup>19</sup>. Dynamic

staining of memory markers on cells cultured in micro-wells can complement such studies. By continuous observation of the differentiation path over time in individual cell clones, discrepancies between different models may be resolved. This type of analysis may also facilitate the construction of more accurate models of memory formation, as time constants and dynamics of memory marker expression levels can be obtained at each phase of clonal expansion.

### **Deciphering the source of initial variability**

A number of mechanisms can contribute to the observed variability in fate between expanding clones. Existing models assume a homogenous starting population, which diversify due to cell intrinsic stochastic or deterministic events. However, initial heterogeneity of the founding cell population can also contribute to their varied response. For example, naïve T cells express heterogeneous levels of the T cell antigen receptor, and these may modulate the cell's quantitative response to an antigen, and lead to different rates of proliferation and differentiation. Similarly, proliferation may be affected by levels of other markers that have heterogenous expression profiles, such as CD5, which was shown to correlate with the level of response of naïve T cells to antigens<sup>48,49</sup>. Live cell imaging in microwell arrays provides a unique system for studying the correlation between initial cell state and the resulting outcome of its direct progeny. Cells can be pre-stained for markers of interest that characterize their initial state, seeded in micro-wells and traced over several days. The initial state of the cell can then be correlated to various properties of its progeny, such as division and death times, cell size, motility and subsequent patterns of gene expression (Figure 3A). We demonstrate this by evaluating correlations between initial levels of CD3 and CD5 on naïve CD4<sup>+</sup> T cells and the resulting proliferative response of these cells following TCR stimulation. Our results indicate that higher initial levels of CD3 result in larger final cell numbers (Figure 3C-D). Initial CD5 levels, on the other hand, did not markedly affect clonal expansion in this setting (Figure 3E-F).

### **Studying intercellular interactions and collective cellular behavior**

The in vivo environment in which T cells and B cells expand is complex, and includes a large number of interacting cells that communicate with each other. These intercellular

interactions affect the fate of expanding lymphocyte clones, and can introduce another layer of variability to their responses. Consider the activation of naïve T cells in lymph nodes as an example. Within lymph nodes, T cells scan dendritic cells (DC) for a cognate antigen. Upon antigen recognition, T cells will stop and establish longer lasting interactions with DCs, allowing cells to communicate by direct contact and also by secretion of cytokines. Thus, a DC can influence the fate of a bound  $CD4^+$  T cell by secreting cytokines that affect its differentiation, or by regulating asymmetric cell division<sup>20</sup>. Upon proliferation, T cells can move to other parts of the lymph node, for example into germinal centers, where they continue to proliferate while encountering new interaction partners that can further influence their fate.

Homotypic interactions also play important roles in shaping lymphocyte responses. Interactions between expanding clones, for example those responding to different epitopes of a pathogen, can lead to phenomena such as clonal competition, clonal dominance, and clonal cooperation<sup>2-6</sup>. Interactions between and within expanding T cell clones are further enhanced by the formation of long-lived T cell clusters<sup>50</sup>. These clusters are suggested to play important roles in T cell activation and in the formation of protective immunity in  $CD8^+$  T cells<sup>25,26</sup>. Such interactions can be mediated by cell-cell contacts and also by autocrine and paracrine feedbacks by secreted cytokines, whose levels can be elevated considerably within clusters<sup>51</sup>. Recent theoretical and experimental work show how interactions between expanding T cells that are mediated by the pleiotropic cytokine IL2 can lead to competition, cooperation and homeostasis<sup>2,27,29</sup>. Regulatory T cells can also modulate T cell expansion through consumption of IL2<sup>28</sup>. As IL2 is secreted by the T cells themselves, these interactions lead to collective behaviors, which may differ considerably from the responses of individual, non-interacting cells.

MWAs are a useful tool for evaluating effects of intercellular interactions as cells are kept in fixed locations throughout the experiment. As an example, it is possible to compare the response of cells in microwells that originally receive a different number of cells by random seeding. This approach was used to investigate the influence of T cell numbers on  $CD4^+$  differentiation and division, showing that the number of cells in the well has a significant influence on both the probability of cell death and on the probability of cell division, which gradually increased with initial cell number<sup>32</sup>. This

effect was attributed to local interactions mediated by IL2 between cells in the same microwell. Microwells can also accommodate cells of different types, allowing for the study of more complex multicellular systems. MWAs provide some advantages over other manifestations of micro-fluidic chips designed specifically for cell pairing<sup>52,53</sup>, as they are easier to design, manufacture and use, are compatible with standard cell culture methods, and provide good statistics since hundreds or thousands of micro-wells can be monitored in parallel. This approach was used to study cytotoxicity of human CD8<sup>+</sup> T cells<sup>35,54</sup>, as well as killing of target cells by NK cells<sup>55,56</sup>. Studies of T cell clonal expansion in MWAs can be extended to include antigen presenting cells, further mimicking physiological interactions. We demonstrate feasibility of this approach by co-culturing CD4<sup>+</sup> T cells from OT-II mice, which express a transgenic TCR that recognizes a peptide of the ovalbumin protein, with dendritic cells that present the cognate antigen to these T cells (Figure 3G). Upon peptide recognition, T cell proliferation is observed in some, but not all microwells that contain both cell types. The proportion of wells containing proliferating cells increases with increased peptide concentration (Figure 3H). Heterogeneity is observed in the rate of proliferation and in the resulting clone size, similar to experiments in which antiCD3/antiCD28 stimulation was used. These systems can be further extended to include more cell types, or other molecules of the natural microenvironment that coat the surface of the device, providing “synthetic immune niches” of increasing complexity<sup>36</sup>.

### **Summary**

Heterogeneity within and between clones of expanding lymphocytes is becoming established as an important characteristic of natural immune responses. Yet, the mechanisms that lead to observed variability, the respective roles of stochastic and deterministic processes, and the functional implications of the resulting heterogeneity are still controversial<sup>17,18</sup>. We reason that the disparity between the heterogeneous and stochastic responses of individual cells and the robust responses of cell populations can be reconciled through experimental studies of the dynamic responses of single cells followed under controlled conditions, combined with mathematical modeling. Current techniques do not allow for continuous tracking of individual cells in vivo over extended

periods of time. Thus, in vivo studies rely on either live cell imaging over shorter time periods, or single cell flow cytometry at discrete time points. We suggest that ex-vivo cell cultures under well-defined conditions can provide missing information about the dynamics of cellular responses, as well as the roles of intercellular interactions in shaping cellular behavior. Such studies are well-suited to the study of stochasticity in cell fate choice and the potential contributions of instructive cytokine mediated signaling and intercellular interactions. By providing continuous quantitative measurements of multivariate parameters, live cell imaging in micro-well arrays can be used to accurately constrain mathematical models describing clonal expansion. Such models in turn generate predictions regarding the behavior of the system under new conditions. The resulting virtuous cycle of computation and experiment will lead to a deeper and better understanding of the complexities of the vertebrate adaptive immune response.

### **Conflict of interest**

The authors declare no conflict of interest.

### **Acknowledgements**

NF acknowledges support by the Israel Science Foundation (grant no. 1254/11), and the I-CORE Program of the Planning and Budgeting Committee and the Israel Science Foundation. BC holds a Weston Visiting Professorship at the Weizmann Institute.

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### **Figure legends**

**Figure 1: Methodologies for studying clonal expansion.** Left) **Following cell populations** in vivo or in bulk culture allows quantitative measurements of the extent of proliferation in response to different antigens, as well as allowing monitoring the differentiation state of the population. Single cell analysis, e.g. by flow cytometry, provides information about heterogeneity in cellular responses. However, it is not possible to distinguish the extent of proliferation and the state of differentiation of individual clones of cells. Middle) **In vivo tracing of single cells** enables measurements of the extent of proliferation of individual clones directly. Thus, interclonal and intraclonal heterogeneity are resolved. It is also possible to link gene expression and differentiation to the size of the clone. These methods enable the investigation of differentiation plasticity – that is, can one progenitor cell give rise to many types of differentiated cells or does each progenitor have only one differentiation fate. The study of sequential differentiation events is also made possible, and the fraction of clones in each state can be directly measured over different discrete time points. This data can then be used to determine the order of differentiation events using mathematical modeling. Right) **Live cell imaging of single cells ex vivo** provides continuous quantification of dynamic gene expression as well as timing of divisions and death events in individual clones. It further allows for identification of correlations between different properties such as division or death times of sibling cells, or symmetric vs. asymmetric acquisition of cell fate, which affect clonal heterogeneity. Such multivariate characterization is invaluable for in depth understanding of diversity in clonal expansion, and the mechanisms by which it is generated. Live cell imaging in well-defined synthetic environments enables also the study of intra- and inter-clonal interactions.

**Figure 2: Quantitative and continuous characterization of cell expansion and differentiation by time-lapse imaging of single cells in microwell arrays.** A) **Schematics of a typical experiment using micro-wells:** micro-well arrays (MWA) are placed in glass-bottom tissue culture plates. Lymphocytes are isolated and seeded in a random fashion on the MWA, in which they are activated and followed by live cell imaging for several days. Parameters that characterize cell state, such as division and death times, and expression dynamics of different genes, are extracted from imaging data for a large number of clones in parallel. B-left) Images from a time-lapse movie, showing one micro-well within an array, which initially contained a single naïve CD8<sup>+</sup> T cell. Expression levels of CD62L (red) and CD27 (green) were measured using Live Staining. Cells were activated using antiCD3/antiCD28 activation beads and imaged for 72h. B-right) Changes in fluorescent levels over time, integrated for the entire clone of cells that developed within the micro-well, as extracted from the movie. C-H) **Correlation between cell expansion and differentiation.** C) Relative cell area in wells which contain a single clone that started from one cell, same experiment as in B. Each row corresponds to one micro-well / clone. The relative area is defined as the area of cells at each time point divided by the area of the micro-well. Increase in cell area is indicative of the rate of expansion, which varies between different clones in the experiment. D) CD62L expression over time for the same micro-wells as in C. CD62L is down-regulated after TCR activation, down-regulation occurs at different times for different clones. E) CD27 expression over time for the same micro-wells as in C. CD27 is upregulated over time. Induction time is rather homogenous among clones. F) Clone size at 72h. Cell nuclei were stained with Hoechst 33342 and cell number was evaluated using stain intensity. G) A plot of CD62L down-regulation time (time to reach half-Max value) vs. rise-time in cell area (time to reach half-Max value). Each dot represents one clone of expanding cells. Most clones show a positive correlation between the two times, that is, late down-regulation of CD62L for clones that expand slower. However, a fraction of clones down-regulate CD62L early on, although they proliferate slowly. H) A plot of CD27 up-regulation time vs. rise-time in cell area. No correlation is observed between CD27 up-regulation and expansion. I-L) **Statistics of cell division times in micro-wells.** I) Distribution of division times of precursor cells (CD4<sup>+</sup> naïve T cells). J) Distribution of division times of daughter cells. K) Correlation between mother and daughter division times ( $R^2 = 0.3$ ). L) Correlation between division times of two sibling daughter cells ( $R^2 = 0.84$ ). Correlation between siblings is high, as observed also in other systems of expanding lymphocytes<sup>23,33</sup>, suggesting that division times are governed by factors inherited from the mother cell.

**Figure 3: Initial variability and intercellular interactions studied using live-cell imaging in MWAs.** A) Schematics of an experimental setup designed to study the influence of initial cell state on subsequent cellular events. Lymphocytes are extracted

and stained for markers that characterize heterogeneity in the starting population. Cells are then seeded into a MWA, are activated and followed by live cell imaging. Parameters that characterize cell state are extracted from imaging data for a large number of clones in parallel. B) Naïve CD4<sup>+</sup> T cells were isolated, stained using fluorescently labeled antiCD3 (red) and antiCD5 (yellow), and seeded in a MWA. Initial level of CD3 (C) and CD5 (E) for each cell was evaluated by fluorescent imaging. Subsequently, cells were activated by antiCD3/antiCD28 microbeads, and were imaged for 72h. Final cell numbers in each micro-well were evaluated using nuclear staining. D) Final cell number positively correlates with initial level of CD3. F) Final cell number weakly depends on initial level of CD5. G - H) Co-culture of TCR transgenic CD4<sup>+</sup> T cells and dendritic cells in a MWA. G) Images from a representative micro-well harboring DCs pulsed with the cognate OVA peptide (blue arrows) and OT-II T cells (green arrow). T cell proliferation is observed. H) Cell area was evaluated in each micro-well that started with one T cells, after 72h of culture. Target peptide is required to induce T cell proliferation. Response is observed in only part of the micro-wells, and final cell number is very heterogeneous. Both the fraction of responding cells and the average cell area show a weak dependence on antigen dose.

**Figure 1:**

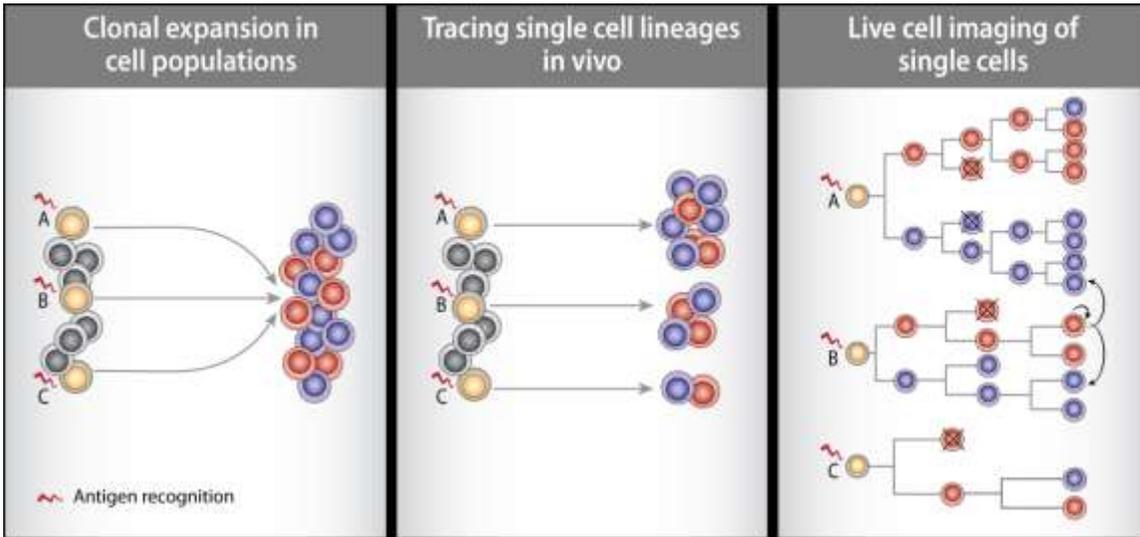


Figure 2:

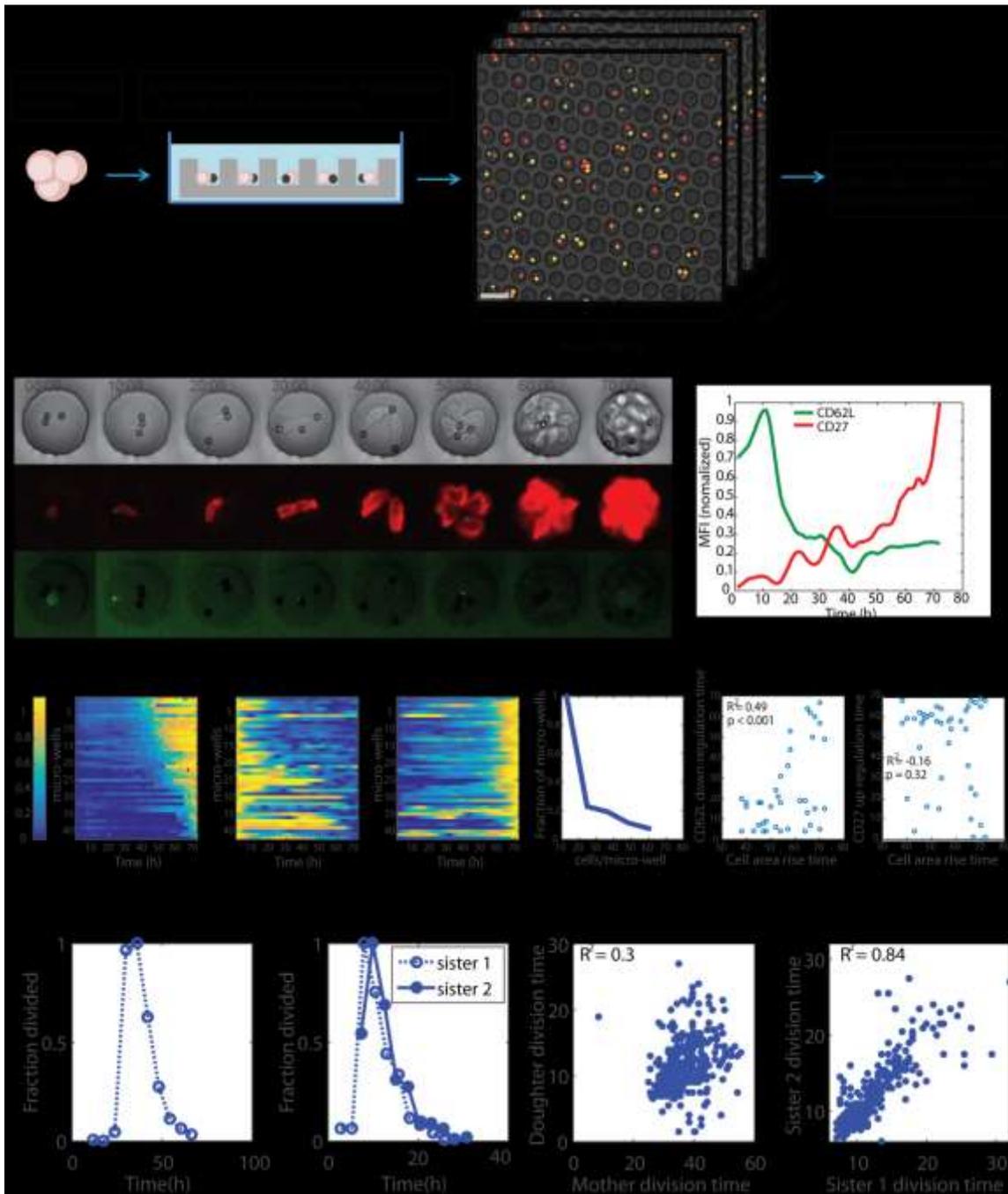


Figure 3:

