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Single-cell technologies for profiling T cells to enable monitoring of immunotherapies

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Abstract

Immunotherapy relies on the reinvigoration of immune system to combat diseases and has transformed the landscape of cancer treatments. Clinical trials using immune checkpoint inhibitors (ICI), and adoptive transfer of genetically modified T cells have demonstrated durable remissions in subsets of cancer patients. A comprehensive understanding of the polyfunctionality of T lymphocytes in ICI or adoptive cell transfer (ACT), at single-cell resolution, will quantify T-cell properties that are essential for therapeutic benefit. We briefly highlight several emerging integrated single-cell technologies focusing on the profiling of multiple properties/functionalities of T cells. We envision that these tools have the potential to provide valuable experimental and clinical insights on T-cell biology, and eventually pave the road for the discovery of surrogate T-cell biomarkers for immunotherapy.

Graphical Abstract

Introduction

Immunotherapy has revolutionized the treatment of cancer and relies on utilizing the patients' immune system and its anti-cancer properties for therapeutic benefit [1,2]. This approach is fundamentally different from chemotherapy and even targeted therapy, both of which depend on the ability of the drug to kill the tumor cell directly [3].

Immunotherapeutic treatment is based on the recognition that there is a failure of the host

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Conflict of interest statement.

The authors declare no conflict of interest.

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immune system to control the tumor adequately, and that the goal of treatment is to facilitate resetting the dysregulated balance to enable eradication of the tumors via the host immune system [4–6]. In other words, the treatment does not work to directly kill the tumor cells but instead tries to reinvigorate the immune system to get rid of the tumors. One of the primary objectives of this approach, akin to vaccination, is the ability to establish immunological memory of the tumor, thereby enabling the immune system to seek and destroy metastases anywhere in the body and enable long-term control [7].

Although utilizing the immune system for therapeutic benefit has been around for quite some time, and proteins such as cytokines (*e.g.* interleukin-2) [8,9] and a suite of monoclonal antibodies (anti-CD20, anti-EGFR, *etc.*) [10–12] have been used clinically over the last two decades, two newer approaches to treatment — the inhibitors of checkpoint molecules [13], and the adoptive transfer of genetically modified T cells [14], have made substantial advances in the clinic. After decades of frustration with the 5-year survival rates of chemotherapy, these newer forms of immunotherapeutic treatment have altered the treatment landscape and have facilitated durable and lasting remissions in subsets of patients [15]. Both classes of treatment, immune checkpoint inhibitors (ICI) and adoptive cell transfer (ACT), critically rely on the functionality of a particular subset of lymphocytes within the immune system — the T cells. ICI aims to reinvigorate T cells and activate them to attack tumor cells and has shown clinical efficacy in various tumors, albeit in only ~20% of patients [16,17]. ACT, on the other hand, delivers *ex vivo* expanded (and/or genetically modified) T cells as the therapeutic and has shown complete responses in leukemias (response rate 70%) [18–22].

The introduction of immunotherapeutic molecules as drugs has facilitated new challenges and opportunities for engineers. While the potency of small-molecule-based therapies can be mapped to their mechanism of action (binding/inhibiting appropriate proteins) facilitating tumor cell killing [23,24], understanding the efficacy of ICI or ACT is a significant challenge since the mechanism of action is neither simple nor wholly defined [13,25–27]. The origin of this challenge can be mapped to our inability, to define in a comprehensive manner, all of the different T-cell functionalities that can contribute to their efficacy. T cells are essential players in the adaptive immune systems and can recognize cognate antigen through their T cell receptor (TCR) [28]. T cells bearing TCR specific for foreign or non-native peptides displayed in the context of human leukocyte antigens (HLA) get activated and can undergo a process of programmed differentiation depending on the availability of other accessory molecules including cytokines within the activating environment. Unlike antibodies, the TCR itself does not undergo somatic hypermutation subsequently, and hence can be considered a barcode to identify populations of clonally related T cells [29–31]. T cells are capable of many different functions including cytotoxicity, cytokine secretion, proliferation, and migration, which are determined by multiple cues from intrinsic properties of T cells and its environmental factors. The relative importance of these functions in defining clinical benefit is only partially understood and confounded by the differentiation status of the T cell (naïve, stem-cell-like central memory, central memory, effector memory and effector) [32,33], or by their functional status (polyfunctional, anergic, or exhausted). It is thus apparent that the availability of methods that can map all of these properties onto the same T cell will advance our understanding of the efficacy of immunotherapeutic treatments.

From the perspective of the ACT, the availability of precise definitions on the properties that need to be engineered into the T-cell infusion product will facilitate consistent biomanufacturing of therapeutic products [34]. It is thus clear that immunotherapeutic treatments stand to benefit from single-cell technologies that can map the complexity of T cells. While the vast majority of advances in immunotherapeutic treatment have focused on oncology, the principles of modulating the immune system are likely to find broad applicability in other infectious diseases and autoimmunity, as well.

Single-cell technologies have attracted researchers' attention for several decades, and there is an increasing trend for scientists to develop more accurate and sensitive, higher-throughput and automated single-cell characterization tools. These approaches allow the detection of details that cannot be revealed using traditional population-level assays [35]. Generally, these single-cell technologies are designed to capture cellular information from either the genome, transcriptome or more recently the proteome level [36]. While some assays like flow cytometry (FC) have been standardized and used even in clinical settings [37], some of the more recent single-cell technologies like mass cytometry (MC) [38], and single-cell RNA sequencing (scRNA-seq) [39] have been recently commercialized. Despite this, however, the vast majority of tools are designed in the research setting, and recent advances have enabled the integration of approaches from different omic dimensions to be able to quantify cell features simultaneously [40].

In this review, we briefly highlight several types of emerging single-cell technologies, mainly focusing on technologies that monitor multiple features (function, transcripts, phenotype, *etc.*) in the context of T-cell characterization. We believe that analyzing T cells at single-cell resolution will provide valuable insights on both experimental and clinical investigations, and has the potential to improve the clinical outcomes of T-cell based therapy. Furthermore, the development of multiplexed single-cell interrogations tools to explore the phenotypical and functional correlations within heterogeneous T cells populations can reveal the underlying biological networks, eventually paving the way for both a better understanding of T cells and delivering surrogate T-cell biomarkers for immunotherapy.

Protein detection from single-cell

Single cell western blotting (scWB)

Similar to the standard western blotting methodology, this approach includes protein separation based on both the affinity between the antibody and the target protein, and the relative size of protein thus minimizing concerns about antibody-cross reactivity (Figure 1A). By the application of open microwells on a polyacrylamide gel coated glass slide, single cells were deposited into individual wells, subsequently lysed, subjected to gel electrophoresis, immobilized by UV-light, and the protein detected by immune-probing. By repetition of antibody-stripping and re-probing, it could detect up to eleven different proteins across thousands of single cells in the same experiment [41–43]. By utilizing a combination of lab-on-a-disc cell device and the scWB analysis, it was possible to quantify protein from less than one hundred cells [44]. The same group further developed an approach termed single cell isoelectric focusing (scIEF) using isoelectric point (pI) difference to separate protein isoforms [45]. In this work, they reported ten cells were analyzed in the same chip as

a proof-of-concept; however, the throughput can theoretically be scaled up. ScWB can be combined with flow sorting [46] or on-chip cell phenotyping [42]. This approach can be beneficial for direct measurement of proteins in a single cell, especially when the number of cells available is limited, allowing the validation of known biomarkers in a multiplexed fashion while maintaining single-cell resolution.

Integration of protein detection and transcriptional profiling of single cell

Flow cytometry

FC has been widely adopted for several decades to characterize the phenotype of cells and the intracellular molecules across millions of cells. It can detect up to around 17 parameters simultaneously, which is determined by the availability of fluorescent dyes [47]. Recently, Nicolet *et al* were able to simultaneously profile the expression of primary human T-cell cytokines (IFN- γ , IL-2, and TNF- α) at both the protein and mRNA transcript level via integration of fluorescence *in situ* hybridization (FISH) and a flow cytometry-based platform [48]. This work paved a road for finding the correlation between cytokine secretion and mRNA transcripts within the same single cell.

Mass cytometry

To improve the multiplexing capacity of cytometry, heavy-metal tagged antibodies are used in mass cytometry (MC). This strategy enables the quantification of more targets on single cell simultaneously, including surface phenotypic marker characterization, intracellular protein detection, cytokine secretion, transcription factor expression, and mRNA transcripts expression [26,49–55]. Frei *et al* developed a method called PLAYR (proximity ligation assay for RNA) and demonstrated that this approach was able to quantify multiplexed mRNA transcripts and protein via flow cytometry or mass cytometry simultaneously [51]. The oligonucleotide labeled-fluorescence or metal tags were used to detect target transcripts. The authors validated this method by detection of 8 different mRNA transcripts and 18 proteins (cytokine + surface molecules) in LPS-stimulated PBMC for various stimulation times, and the results suggested the most LPS-responding cells were likely to be a CD14⁺ phenotype. Frei and colleagues expected the theoretical upper limit in the number of detected targets could be as high as 40 if combined with MC. The disadvantage of MC is that unlike FC, it is sample destructive, and thus, it is not possible to sort single cells for downstream analyses like RNA-seq.

Both FC and MC are well-developed technologies and can directly detect proteins from millions of single cells but are restricted to providing snapshots since it is not possible to track the same cell longitudinally using these methods. Despite these disadvantages, however, FC and MC are robust knowledge-based methods to identify subsets of T cells directly from tumors and hence will play an essential role in tracking the efficacy of immunotherapies.

Single-cell PCR

Unlike the PLAYR method that utilized the mass tag or fluorescent tag to capture transcript or protein abundances, other studies relied on the usage of DNA as a label to detect proteins.

Although initially the profiling of mRNA and protein was achieved by splitting the cell lysate to two parts and characterizing each of them separately [56,57], Genshaft *et al.* presented an approach that combined the detection of protein and mRNA from same mammalian cells in a single reaction chamber in a parallel manner [58]. Modified proximity extension assays (PEA) method was used in this technology for protein detection. For each protein of interest, there were two different single-stranded oligonucleotides-labeled antibodies to detect the target protein. The 3' end of DNA labels of this antibody pair were complementary to each other, as a result, DNA labels would hybridize once both antibodies co-localized on the target protein. The extension of DNA label complex and reverse transcription of RNA from the same cell happened simultaneously by utilizing reverse transcriptase also as DNA polymerase, followed by qPCR (FluidigmTM C1 system) to quantify protein expression and RNA abundance. By applying this approach to study protein and mRNA abundances in the PMA-stimulated MCF7 cells, they found that the correlation of mRNA and protein was variable among genes or time points: highly-expressed genes were more correlated with the corresponding protein expression in untreated cells but after simulation the lowly-expressed genes with high cell-cell variance showed the largest correlation.

ScRNA-seq

ScRNA-seq, a rapidly-growing technology can provide unbiased, high-dimensional genome-wide transcriptomic profiling of individual cells, and has emerged as a robust method to facilitate the discovery of novel cellular status [59], and provide biological insights [31,60,61]. ScRNA-seq has been extensively reviewed elsewhere [39,62–64], and we will only highlight combinations of scRNA-seq with other kinds of single-cell assays [39,62–64].

Researchers have developed several algorithms to utilize scRNA-seq data to reconstitute T cell receptor information. One advantage of obtaining TCR information at single-cell level is that the possibility to acquire the pairing detail of TCR chains ($\alpha\beta$, $\gamma\delta$). Computation approaches, such as TraCeR [65], scTCRseq [66], VDJpuzzle [67], work quite well with transcriptomic profiling results obtained from full-length mRNA transcripts. More recently, the TRAPeS pipeline was reported to enable TCR information extraction from short-read (25–30 bp) sequencing data [68]. Combining transcriptomic profiling and TCR profiling at single-cell resolution, the clonal expansion of exhausted or dysfunctional T cells was found in tumor sites, indicating the reinvigoration of T cell function may recover its anti-cancer functionality [31,61]. Owing to these emerging computational pipelines, developmental trajectories of diverse T cell population can be deciphered, holding the promise of investigating the antigen-specific T cells functions in response to diseases, and also to identify the diversity of T-cell responses within the tumor microenvironment.

Stoeckius *et al* and Peterson *et al* recently reported two closely related methods (CITE-seq and REAP-seq) for simultaneous detection of mRNA and protein [69,70] (Figure 1B). Both methods utilized a combination of unique oligonucleotide barcodes and poly (dA) sequence for indexing antibody (but using different linkers) thus enabling the detection of multiple proteins along with transcripts. Extension of DNA labels of antibodies and reverse transcription of mRNA transcripts could be achieved in the same reaction by taking

advantage of the DNA polymerase function of reverse transcriptase. These two methods can be readily adapted to different high-throughput scRNA-seq platforms. Another similar technique that can be expanded to demonstrate the same capability is called Abseq (Figure 1D), which utilizes a combination of DNA-labeled antibody and droplet microfluidics [71]. One disadvantage of all three of these approaches is that the information about the spatial distribution of proteins is lost. An orthogonal method, Seq-Well (Figure 1C), takes advantage of arrays of microwells instead of droplets. The cell lysis and reverse transcriptions of mRNA are accomplished on-chip by sealing single cells and individual barcoded capture beads [72]. This assay is compatible with on-array imaging cytometry for resolving the phenotype of cells from complex samples and has the potential to obtain more information from a limited amount of samples using a single platform.

Unquestionably, the integration of transcriptomic and proteomic profiling mentioned above on the same single cell can characterize cellular response to a perturbation in a more accurate, unbiased way. However, these approaches require cell fixation or cell lysates, which exclude the possibility for tracking the dynamic transcriptomic and proteomic changes in the same cell. Although it has the advantage of being able to profile the complete transcriptome, the abundance of lowly expressed transcripts like transcription factors remains a challenge and requires pooling of data when the magnitude of change is also small. Recent reports have aimed to improve the analysis algorithms and to extract more information out of the data [73–76]. Since the cells are lysed to retrieve the mRNA, scRNA-seq ideally provides a snapshot of the cell state, inferred by the transcript profile. Immune gene signatures could be obtained from sequencing results without *prior* knowledge, making it ideal for the discovery of candidate biomarkers in an unbiased manner. There are disadvantages of this approach including the lack of correlation between mRNA and protein for some genes [58], the inability to directly detect post-translational modification of proteins, and a complete lack of protein localization information. Thus, an ideal implementation of scRNA-seq would be in combination with another method that directly profiles biological function.

Integrated platforms to monitor dynamic T-cell behavior and polyfunctionality

Immune cells, specifically T cells, demonstrate a variety of dynamic behaviors. From the standpoint of studying the therapeutic potential of T cells for adoptive transfer, or for identifying biomarkers of ICI, quantifying the functional status of the T cells will be essential.

Time-lapse imaging microscopy in nanowell grids (TIMING)

The characterization of the interaction between pairs of cells would benefit the understanding of how cells interact or cooperate with other cells, and help the discovery of underlying mechanisms of dynamic cell behavior. Microfluidic devices have the potential to dynamically monitor cell-cell interaction in a high-throughput manner in combination with live cell microscopy. TIMING (Figure 1E), a microwell-based platform, was reported to be able to dynamically monitor cell-cell interaction, cytotoxicity, cell motility and cell survival

simultaneously [77–79]. Additionally, it can integrate real-time cytokine profiling by bead-based cytokine sensors [80] or gene expression profiling by single cell retrieval via micromanipulator [81] due to the non-destructive feature of this assay. Similarly, it has also been reported that droplets can be used to co-encapsulate the two types of cells before docking to the microwells [82]. This microwell-based device was compatible with live cell imaging analysis, allowing the dynamic monitoring of cell morphology, behavior, and fate. Another recent technology, DropMap, utilized droplets to dynamically profile antibody secretion from antibody secreting cells (ASCs) [83]. Although the rate of secretion of cytokines is much lower than immunoglobulin secretion from ASCs, in principle the DropMap technology can be adapted to monitoring cytokine secretion from T cells.

Single cell barcoding chip (SCBC)

Single cell barcoding chip, pioneered by the Heath and Fan groups, is able to quantify multiple proteins from the same cell, based on the fluorescence readout and on-chip calibration (Figure 1F). SCBC consists of a collection of microchambers on the microfluidic chip (from several hundred to several thousand) to confine single cell or two cells, and one of the surfaces of the microchamber contains barcode-like patterned antibody arrays for protein capture and further detection [84–86]. Apart from protein detection, this approach entitled the monitoring cell movement of the single-cell pair along with the protein secretion [84]. Built on a similar concept, beads-on-barcode antibody microarray (BOBarray) was developed to quantify released proteins from a single cell confined in the individual well, but with modification of protein detection strategy: color-coded and sized-coded functionalized microbeads were coated on the glass slide instead of patterned antibody arrays to minimize the size of the microfluidic device [87]. SCBC technology is amenable of up to around 40-plex protein detection from a single cell and only need a small sample amount as an input; however, due to its intrinsic design, it was not designed to study dynamic or real-time protein secretion.

The advantage of these function-based single-cell assays like TIMING and SCBC is that they have the potential to reveal heterogeneity of complex biologies like motility (reflection of the homing ability of T-cell to find tumor), cytotoxicity (representative of tumor-killing functionality) or cytokine secretion (communication with other immune cells), which are potential predictive biomarker candidates of T cells-based immunotherapy, especially combined biomarkers that require simultaneous measurement of different perspectives of T-cell functionalities. One of the disadvantages of these approaches is that unlike FC/MC that are available as part of core facilities, microfluidics often requires unique expertise and infrastructure to be able to execute these assays. As mentioned above, since the ability to retrieve cells of interest has been demonstrated for at least the TIMING assay, the ability to integrate functional and transcriptional profiling at single-cell resolution might provide the in-depth insight required for defining the efficacy of immunotherapies.

Challenges

Identification and validation of biomarkers

A lot of effort has been devoted to obtaining the predictive biomarkers for ICI and ACT, and candidate biomarkers are showing promising results in multiple clinical trials. Biomarkers could be in various and different formats: tumor infiltrating lymphocyte density at tumor sites, immune checkpoint expression on tumor cells, neoantigen/mutation load of tumor cells, serum protein/antibodies, circulating tumor cells, lymphocyte counts, T cell clonality, *etc* [88–93]. However, validated clinically actionable biomarkers for predicting the clinical outcomes of ICI/ACT are still lacking. The ligand of immune checkpoint expression of certain tumor samples is considered as one of the promising biomarkers for ICI; for instance, PD-L1 qualitative immunohistochemical assays were FDA-approved but with suboptimal sensitivity and specificity [93]. Many factors may contribute to the inconsistent performance of candidate biomarkers including the complexity of the anti-tumor response, the heterogeneity of tumor or/and patients, the inducible and non-static property of biomarkers, the suboptimal specificity and accuracy of assays. It is evident that the discovery of biomarkers with high accuracy and sensitivity will enable better patient selection into the 3000+ immunotherapy clinical trials currently underway. Since T cells are considered the major effector cells in ICI and ACT, it is not surprising that some T-cell based predictive biomarkers have been discovered [52,94]. Although profiling T cells directly from the tumor site is more indicative of T-cell functional status within the tumor, peripheral blood is a more accessible compartment with limited inconvenience to the patients. For this reason, parallel efforts are devoted to studying T cell within the blood to determine if the changes within these T cells can be used to infer changes in T-cell functionality as a result of treatment [52,94]. Unfortunately, however, T cells demonstrate a variety of dynamic behaviors, and thus mapping these to the same T cells, at single-cell resolution is a challenging problem (Figure 2).

Choice of assay

The choice of the appropriate assay to define T-cell functionality is defined by a combination of multiple factors including the number of T cells available, the depth of information being sought, ease of assay standardization, and cost. ScRNA-seq is an excellent choice for the discovery of biomarkers but is still rather expensive for routine implementation. At the other end of the spectrum, FC is considered standard but it requires that the biomarkers have already been discovered using another assay. Presently, at least for monitoring ICI trials, there is no unique way to converge to a single-assay and the choice is often determined by one or more of factors listed above. ACT, on the other hand, should not be limited by cell numbers for at least the infusion product, and not surprisingly more complex assays and dynamic assays have been used to evaluate T-cell functionality [95,96].

Spatial information

All the techniques we have described work with homogenized single cells or single cells in suspension. These methods are ideal and relevant in tumor immunotherapy when profiling single T cells in peripheral blood. Thus, while the comprehensive documentation of the molecular profiles revealed by scRNA-seq is useful for identifying compositional

frequencies of immune cell subsets, they cannot, however, reveal the link between the molecular profile and functional capacity, and how this is impacted by space and time. The tumor microenvironment is a three-dimensional structure composed of different kinds of cells, and it is important to document the spatial localization of immune cells within the tumor microenvironment (TME). A few *in situ* sequencing, proof-of-concept technologies have been demonstrated that can directly map spatial information and transcript profiles [97–99], but it remains to be seen if they can match the depth of transcript profiling afforded by even scRNA-seq. Similarly, FISH based methods that can preserve the spatial information and directly count RNA molecules down to the single-molecule level, have been reported [100–102]. The drawbacks, however, are that even with repetitive cycles of probing different mRNA molecules, the total number of unique mRNA molecules that can be detected is smaller than scRNA-seq and that researchers have to pre-determine the transcripts that are being studied.

Bioinformatics

One of the major and central challenges for realizing the potential and benefit of the next generation of single-cell technologies is matching advances in bioinformatics. As outlined above, the low number of reads per single cell, the ability to differentiate technical and biological variation, amplification biases, batch-effect, all present significant challenges to systematic data analyses. In addition, the ability to integrate single-cell data acquired across different platforms analyzing different kinds of biomolecules and functions is a complex problem, which requires adequate normalization methods and the capability to investigate the correlation among different dimensions of single-cell data [73,103–105]. The identification of conserved signatures of genes, and dimension-reduction-based visualization are the most common methods to extract information from single-cell datasets with high-dimensionality [105,106]. However, the algorithms for prediction of single-cell responses, within heterogeneous cell populations, to perturbation is not well-defined [107]. In other words, while the currently available analytic approaches are mainly focusing on descriptive analyses at single-cell resolution *in vitro*, it remains unclear how to utilize and integrate these single-cell data to accurately predict the behavior and fate of diverse cell populations *in vivo*, especially within TME, eventually serving as biomarkers to predict the clinical outcomes of cell-based therapeutics.

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- of special interest
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Highlights

- Quantitative characterization of dynamic T cell behavior can facilitate identification of biomarkers of responses to immunotherapeutic treatment
- Snapshot single-cell omics has made significant advances but lacks temporal resolution
- Multi-dimensional techniques that integrate dynamic T-cell function with omics at the single-cell level are starting to emerge

Figure 1. Single-cell technologies for multi-dimensional characterization

(A) Workflow of scWB adapted from ref [41].

Pore-gradient gel arrays allow thousands of protein electrophoresis separations at single-cell resolution within 1 mm separation distance on a microscope glass slide. Single cells are loaded to individual microwell by gravity and are chemically lysed *in situ* on the chip. Once scWB is performed, the gel gets brief UV exposure for protein immobilization. Acid is used for pore size expansion, which facilitates the enhancement of local antibody concentration for immunoprobation. Fluorescence images of scWB for proteins spanning from 25–289 kDa. Closed-up false color fluorescent images represent part of arrays (over 400 lanes).

(B) Workflow of REAP-seq adapted from ref [69].

A droplet containing Ab-Barcodes (AbBCs) coated cells fuse to another discrete droplet which contains cell-barcode beads with primers. The cell is lysed once two droplets fuse, and polyadenylated mRNA and AbBC hybridize with poly(dT) primer and the extension of AbBC and complementary synthesis of transcripts can be achieved by reverse transcriptase in the same reaction. AbBC sequences (~155 bp) and cDNA from mRNA (~500 bp) are separated based on the size difference, and protein and transcript libraries are constructed and sequenced.

(C) Workflow of Seq-Well adapted from ref [72].

The complex tissue is dissociated to single-cell suspension first, and then barcoded mRNA capture beads and cells are loaded onto microwell array by gravity. The device is sealed by a semipermeable membrane to allow lysis buffer change but confine mRNA within the well. Once the beads (containing poly(dT) primers, which including cell-specific barcodes and unique molecular identifiers for each transcript) capture liberated transcripts from an individual cell, the beads are recovered from the array. Reverse transcription of bead-bound

transcripts is performed in bulk, followed by library preparation, sequencing and *in silico* analysis.

(D) Abseq workflow adapted from ref [71].

Cells stained with DNA-conjugated antibodies are isolated in a droplet with unique cell barcoding information, and the linkage of antibody barcode and cell barcode is achieved via overextension PCR. The chimeric DNA products from over 10,000 single cells can be pooled and sequenced in parallel. The single cell protein information will be sorted by the cell barcoding. Unique molecular identifiers are utilized for PCR-bias correction.

(E) Workflow of TIMING (Time-lapse imaging in nanowell grids) adapted from ref [77].

Raji tumor cells and NK cells stained with PKH26 and PKH67 fluorescent membrane dyes respectively are loaded onto a nanowell array, which is immersed in cell culture media containing fluorescent Annexin V as cell apoptosis indicator and imaged for 6 hours by high-throughput time-lapse imaging. Imaging analysis is performed as previously described [79].

(F) Schematic of a single cell barcode chip (SCBC) and representative time-lapse images of an SCBC microchamber containing two cells. Adapted from ref [84].

Top left: schematic of a SCBC microchamber with valve and DEAL (DNA-encoded antibody library) barcodes; bottom left: immune sandwich formation indicates protein detection; top right: representative time-lapse images of an SCBC microchamber containing two cells over 8 hours; bottom right: fluorescent images of patterned barcodes of 5 detected proteins, scale bar = 100 μm .

Figure 2. Integrated and dynamic profiling of T cells

T cells are capable of many different functions and integrate cues from both the cells and soluble factors from the microenvironment to facilitate decision-making. A complete understanding of T cells can be only accomplished by tracking dynamically cell-cell interactions (e.g. synapse formation, cytotoxicity), intrinsic and chemokine guided motility, cytokine secretion, bioenergetics, transcriptome, morphology, differentiation status and proliferation or survival. Assays that are able to provide insights into one or more of these features on the same cells, at single-cell resolution, can provide a deeper understanding of the underlying biology.

Table 1

Selected examples of single-cell technologies that enable characterization of multiple features

	Technology	Throughput	Highlight	Reference
FC/microscopy & protein	scWB	Up to thousands of single cells	<ul style="list-style-type: none"> Combination of microwells and PAGE gel for protein detection based on mass or/and pI Can detect up to 11 proteins on the same cells by antibody stripping/re-probing Compatible with FACS sorting or cell imaging as pre-characterization Re-probing archival sample is possible 	[41–46]
Protein&mRNA	FC	Millions	<ul style="list-style-type: none"> Fusion of Flow-FISH & ICS Quantification of mRNA + protein of three cytokines simultaneously (IFN-γ, IL-2, and TNF-α) 	[48]
	MC	Millions	<ul style="list-style-type: none"> PLAYR Relies on MC (mass tag) or FC (fluorophore) for protein (antibody) and transcript (oligonucleotides) read-out The multiplexing capacity is determined by the available tags (~40 MC) 	[51]
	Single-cell PCR	96 (using Fluidigm™ C1)	<ul style="list-style-type: none"> Leverages the DNA polymerase activity of reverse transcriptase to simultaneously perform proximity extension assays and complementary DNA synthesis in the same reaction Compatible with scRNA-seq platform Demonstrated detection of 96 RNA + 38 proteins 	[58]
scRNA-seq&TCR-seq	TraCeR, scTCRseq, VDJ Puzzle, TRAPeS	Depends on throughput of scRNA-seq	<ul style="list-style-type: none"> Extract TCR information from scRNA-seq results Provide both transcriptional profiling and clonality of single T-cell 	[65–68]
scRNA-seq	scRNA-seq	Thousands of single cells	<ul style="list-style-type: none"> CITE-seq (10 surface proteins), REAP-seq (82 proteins) Oligonucleotide with poly A tail as unique antibody barcode Use reverse transcriptase as DNA polymerase to extend antibody barcode and reverse transcription of mRNA simultaneously Compatible with current scRNA-seq platform 	[69,70]
	Abseq	>10,000 cells	<ul style="list-style-type: none"> Detection protein via DNA-labeled antibody to increase multiplexing capacity Each antibody also has UMI sequence for PCR-bias correction Compatible with current scRNA-seq platform 	[71]

	Technology	Throughput	Highlight	Reference
			<ul style="list-style-type: none"> Theoretical limit of detected protein is determined by sequencing depth and availability of antibody 	
	Seq-Well	~15,000 cells	<ul style="list-style-type: none"> Co-capture cells and transcripts-capture beads within individual microwells On-chip lysis, reverse transcription in bulk Compatible with on-chip imaging cytometry Transcriptomic profiling is done by scRNA-seq 	[72]
Integration: cell-cell interaction, protein, etc	TIMING	20,000 cells	<ul style="list-style-type: none"> Co-culture lymphocyte and target cell on the same individual microwell Time-lapse microscopy live cell imaging Integrated with real-time cytokine secretion and cell retrieval for gene expression profiling 	[77–81]
	Droplet	~1,000 events	<ul style="list-style-type: none"> Similar to TIMING Co-encapsulate two types of cells within a droplet Droplet docking into individual microwell 	[82]
	SCBC	Up to several thousands of cells	<ul style="list-style-type: none"> Single cells or cell pairs are isolated in individual microchambers Cell-cell interaction can be investigated Detection antibody coated surface is detachable for analysis Up to 45-plex protein detection including secreted proteins 	[84–86]
	BOBarray	Several thousands of cells	<ul style="list-style-type: none"> Similar to SCBC, but use antibody-coated beads as protein sensor Miniaturized device achieved by combination of bead size and fluorophore combination: 4 bead size $\times$ 3 color = 12-plex 	[87]