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Single cell analytic tools for drug discovery and development

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Abstract

The genetic, functional, or compositional heterogeneity of healthy and diseased tissues presents major challenges in drug discovery and development.[1-3] In cancers, heterogeneity may be essential for tumor stability,[4] but its precise role in tumor biology is poorly resolved. This challenges the design of accurate disease models for use in drug development, and can confound the interpretation of biomarker levels, and of patient responses to specific therapies. The complex nature of heterogeneous tissues has motivated the development of tools for single cell genomic, transcriptomic, and multiplex proteomic analysis. We review these tools, assess their advantages and limitations, and explore their potential applications in drug discovery and development.

Introduction

Over the past few years, there have been significant advances in the development of single cell analysis tools. For example, about five years ago, patch-clamping electrophysiology methods[5], fluorescence in situ hybridization[6, 7], flow cytometry[8, 9], and ELISpot[10] assays were about the only single cell molecular analysis tools available. Most of those methods could only analyze between 1-3 molecules from a given cell, although multi-color flow cytometry could capture about a dozen cell surface protein markers[11].

This landscape is rapidly changing, and several technologies to comprehensively analyze the single cell at the molecular-level have now emerged. As examples, single cell tools and methods exist that can assay for reasonably large numbers (>40) of secreted proteins[12], equally large numbers of cell surface markers[13], and elements of phosphoprotein signaling pathways[14, 15]. In addition, single cells can now be analyzed for the genome at focused [16, 17] or high coverage[18], the transcriptome at sparse coverage[19, 20] or the entire transcriptome with moderate[21] or high[22] cell statistics.

Additional reports in which integrated measurements of genes and transcripts[23], limited numbers of proteins, transcripts[24, 25] and genes[26], and panels of proteins and metabolites,[27]

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from single cells have also appeared. Microfluidic methods permit molecular analysis to be correlated with measurements of specific cellular functions (such as motility), or allow the analysis of defined, small populations of cells (i.e. 2-3 cells)[28-30]. Microfluidic designs can also permit cell analysis within highly controlled, custom environments,[31-33] or can allow for non-destructive cell analysis, so that cells identified as interesting, such as B cells producing specific antibodies, can be harvested for further use.[34, 35] Two recent tissue staining methods, in situ RNA profiling via sequential hybridization[36-38], and proteomic analysis via ion beam profiling[39] can enable the analysis of single cells within fixed, intact tissues, with a level of multiplexing that significantly exceeds traditional immunohistochemical staining methods. The level of analyte quantitation varies from measurements that yield copy numbers per cell[22, 36, 40], to relative quantitation between cells. Many of these methods result in relatively new types of data, and so are being integrated with new computational approaches[41-45]. In fact, the development of computational tools that can analyze what are increasingly large single cell data sets is lagging behind the advances in experimental methods.

Although these diverse and rapidly evolving single cell technologies provide remarkable opportunities for drug discovery and development, they also provide a deluge of information for the non-technologist to wade through. This review is therefore intended to serve as a guide for the non-specialist. Here, we describe the state-of-the-art of single cell biology tools for different analyte classes, and discuss the new types of biological information that can be gleaned through the use of these tools, highlighted by 3 illustrative examples. To illustrate the broader application of these emerging technologies, these tools are placed within the context of two classes of cancer therapies. The first is the development and use of targeted inhibitors for treating heterogeneous tumors. The second is cancer immunotherapy, which is an area in which several single cell analysis tools are already playing important roles.

Single cell analysis tools can be grouped according to the measured analytes, i.e. genomics, transcriptomics, proteomics or metabolomics-based approaches, or by a combination of these. It is anticipated that the methods described here will likely emerge in the marketplace within a couple of years, although earlier generation variants are, in many cases, already commercially available as either whole platforms, commercial services, or through purchase of critical reagents.

Single Cell Genomics

The rapid technological advances in DNA sequencing tools have exposed the whole genome, the exome, and the transcriptome for single cell analysis. For single cell whole genome sequencing[16, 46, 47], the genome must be amplified prior to sequencing. In principle, this can be done with PCR-based whole genome amplification (WGA) methods, but such methods are prone to bias because random genes can be over or under-amplified by the nonlinear PCR process[48]. A commonly used alternative is the multiple displacement amplification (MDA) method, which is a technique that utilizes the φ29 DNA polymerase enzyme for DNA synthesis[49], and can amplify DNA isothermally at 30°C. MDA provides an improved representation of the whole genome, but the φ29 enzyme is still a nonlinear amplifier (like PCR), and so can yield bias. Such bias, in turn, makes it difficult to discern copy number

variations (CNVs) and single-nucleotide variations (SNVs), although Dago and coworkers have reported measurements of such quantities from single circulating tumor cells (CTCs) originating from prostate cancer.[50] A second WGA approach, called multiple annealing and looping-based amplification cycles (MALBAC), has been recently reported[51]. MALBAC is designed so that the initial polymerase amplification steps yield an amplicon that, due to complementary sequences incorporated into the 3′ and 5′ ends, cyclizes, and so is not available as a template. This keeps the initial genome amplification process linear, and reduces amplification bias. As a result, CNVs and SNVs can be reliably quantitated at the single cell level. As an illustrative example, MALBEC has been extended to the analysis of CTCs from lung cancer patients.[52] For certain challenging genes, such as oncogenes with multiple variants[53], or the T cell receptor (TCR) α/β genes, nested PCR methods[54, 55] coupled with Sanger sequencing, are used. Recent, highly parallel, multi-step RT-PCR based techniques, coupled with next-generation sequencing tools, now allow such sequences to be determined from many (100 or more) single cells in parallel.[56]

Various target enrichment strategies have been developed to broadly select genomic regions of interest for sequencing.[57] For example, as methods for exome sequencing have become standardized[58, 59], they have been extended to single cell analysis[60]. Exome sequencing involves sequencing the 1% of the genome that is protein coding. This is a relatively cost-effective procedure that yields an enriched data set of highly penetrant variants, such as those that are relevant to genetic disorders, or diseases that exhibit a genetic instability, such as many cancers. Examples of single cell investigations include capturing the genetic heterogeneity of tumors[60, 61] or comparing CTCs with the originating tumor or metastatic lesion.[62] Exome sequencing is a technique of rapidly increasing relevance to immunotherapy, as will be discussed below.

Single Cell Transcriptomics

Although the analysis of gene expression at the single cell level dates back to the early 1990s[63], the field has rapidly advanced over the past 5 years, with RNA sequencing (RNA-seq) f exploiting the success of next-generation sequencing tools.[64] Indeed, RNA-seq has advanced at such a rapid pace that a new report emerges almost every month describing a new set of protocols that enable an increasingly deeper and more quantitative analysis of larger numbers of single cells,[22, 43, 65-68] with applications that range from the analysis of immune cells,[67] CTCs,[69] or capturing the transcriptional heterogeneity of various healthy[20, 70] and diseased tissues.[21] The basic biochemical method is PCR, but the major technical challenges have been to engineer contamination-free methods that can account for PCR bias correction and yield absolute quantitation. This has been best accomplished through the combined use of microfluidic platforms,[66] including nanodrop technologies,[71, 72] and molecular barcoding techniques.[65, 72] The microfluidics character of these approaches implies that individual cells are isolated in volumes ranging from a few tens to a few hundreds of picoliters, and this lends several advantages. First, molecular diffusion times within such small volumes are short, and this can significantly shorten the times required for chemical reactions that are part of the processes flow. Second, the small volume raises the relative concentration of the cellular analytes being investigated, and

lowers the copy numbers of any molecular contaminants. Finally, small volumes limit reagent costs and allow many cells to be interrogated in parallel.

Two very recent quantitative single cell transcriptomic methods - CytoSeq and inDrop - are conceptually similar, but distinct in practice (Figure 1). Fan *et al.*[22] reported on the CytoSeq technique that utilizes dilute cell loading into 20 picoliter volume microwells. Into each well is placed a 20 micrometer magnetic bead that is functionalized with many oligonucleotide primers, each containing a universal PCR priming site, a combinatorial cell label (the barcode), a unique molecular index,[73] and an mRNA capture sequence. All primers on each bead contain the same cell label, but incorporate a diversity of molecular indices. Many mRNA molecules from a lysed cell are captured on a single bead, and all beads are combined for amplification and sequencing. Each sequence carries the barcode (single cell identity), the molecular index (1 index per transcript), and the gene identity, thus yielding a relatively deep, bias-free and quantitative analysis of the transcriptome from many single cells in parallel.

Two droplet microfluidics variants of this barcoding approach for single cell transcriptomics are the DropSeq method,[72] and the (simultaneously published) inDrop approach.[71] The basic concept of droplet microfluidics is to use microfluidic channel designs and flow control to combine oil and water so that the water separates into sub-nanoliter volume droplets separated by oil. Each of those nanodrops can be seeded with a cell, a barcoded microbead (or equivalent), cell lysis reagents, etc., so that each nanodrop comprises a self-contained reaction vessel. Advanced microchip designs allow virtually the entire process, from cell introduction, to delivery of reagents for sequencing, to be automated on a microchip about the size of a microscope slide. The drop-seq method was utilized for the analysis of nearly 45,000 single mouse retinal cells, which is a testament to the scalability of droplet microfluidics, and similar to the capabilities of the CytoSeq method.

A common concern with single cell methods is the relationship between what is measured, and the copy numbers of the analyte that were actually in the cell. Even genetically identical cells, cultured side-by-side, will naturally exhibit significant variations in copy numbers of transcripts, proteins, metabolites, and other analytes (see Box 1).[42, 74] For any analyte, capture efficiency is always an issue, and can be very challenging to quantify. For transcriptomics, this concern is complicated by the fact that the actual mRNA transcript is not measured, but instead it is a cDNA complement, amplified to many copies, that provides the input into the sequencer. Different mRNAs can be differentially amplified, and noise can be amplified along with signal.[48, 75-77] Of course, having a single cell technique that captures the biological heterogeneity of the cells under study, rather than the measurement noise of the technique itself, is advantageous. Various methods have been used to increase and/or characterize the quantitative nature of single cell transcriptomics.[78]

The Unique Molecular Index (UMIs)[65, 73, 74] mentioned in the description of CytoSeq, which is also utilized by both microdroplet methods, is a protocol designed to limit amplification bias by associating a unique molecular signature to each mRNA copy that is captured. A related method was reported by Fu and coworkers.[79, 80] Thus, if 10 copies of a specific transcript are captured from one cell, each will have the same barcode, but a

different UMI, and so the copy numbers of a given mRNA captured is simply the number of unique UMIs for a given barcode. The use of UMIs, while a significant step towards absolute quantitation, does have limitations for counting low copy number transcripts.[74]. A second major issue is that of capture efficiency, which can vary from <5%[74] to ∼20%,[81] and can be assessed by counting mRNA copies using UMIs relative to those recorded using fluorescence in situ hybridization (FISH).[74] Of course, a low capture efficiency will have a correspondingly large variance across many mRNAs, or between different single cells – especially for low copy number transcripts. Thus, quantitating and increasing capture efficiency is an analytical frontier of the field.

Multiplex single cell proteomics methods

Multiplex single cell proteomic methods (Table 1, Figure 2) are classified as either flow[11]- or mass[13] cytometry (CyTOF) tools, or as microfluidic platforms. Each of these methods rely on antibodies, so, unlike mass spectrometry proteomics of bulk samples[82, 83], single cell proteomics methods cannot yet serve as discovery level tools. For the microfluidic platforms, the microengraving technique[35, 84], single cell barcode chips (SCBCs), and single-cell Westerns[85] (scWesterns) yield the most advanced capabilities. A number of alternative approaches, typically with reduced levels of multiplexing, have been reported, including high throughput microdroplet-base screening approaches,[33, 86-89] and some of these are reviewed elsewhere[90].

For analysis and cell sorting based upon cell surface markers, flow cytometry based fluorescent-activated cell sorting (FACS) is the mature single cell proteomics method,[8] and interfaces with almost all other single cell methods described in this Review. FACS is routinely employed to analyze and sort viable cells based upon a half-dozen or more surface markers, and so is tremendously useful for purifying cellular phenotypes for subsequent analysis.

The analysis of cellular function at the molecular level, or the specific influence of drugs on that function, typically requires the analysis of functional analytes, such as phosphorylated kinases or secreted cytokines, apoptotic or proliferation markers, and/or metabolites. In general, these different classes of molecules can require different assay methods. For analyzing functional cytoplasmic proteins, CyTOF is the most mature tool,[15] although SCBCs have emerged with similar and complementary capabilities[14]. ScWesterns, while having origins that can be traced back to single cell gel electrophoresis assays, known as comet assays[91, 92], represent the youngest technology, but one that is perhaps most closely aligned to standard biology practice. Each of these tools has advantages and limitations. For analyzing secreted proteins, SCBCs have the unique capability of capturing large panels (>40) of proteins secreted from viable cells[12]. Microengraving tools capture only a few secreted proteins, but permit kinetic studies of protein secretion from individual cells[93]. For both SCBCs and microengraving, cells that exhibit unique or desirable protein signatures may be further analyzed.[34] CyTOF can capture large panels of ‘secrete-able’ proteins, but protein secretion must be blocked and the cells fixed prior to analysis, and so the detected proteins are not actually secreted, and the cells cannot be further analyzed[13]. For the microfluidics tools, the cells can be imaged *in situ*, so that factors such as cell motility or

morphology can be correlated with the secretion of specific proteins[94]. The microfluidics tools also permit assays on discrete numbers of cells[28, 44, 95]. Since CyTOF utilizes antibody staining of fixed cells, that staining can be done within fixed tissues, permitting CyTOF to be used as a very powerful variant of immunohistochemical staining.[39] Each of these tools requires significant user skill, although that requirement will likely diminish as the platforms advance.

As with transcriptomic methods, quantitative assessment of single cell protein levels is an increasingly important issue. All single cell proteomics methods utilize antibodies as the dominant detection technology. A recent publication provided a protocol for establishing a clear, quantitative metric for antibody performance, [96] and raised serious questions about whether a given antibody even detects its intended target. Of some 1124 antibodies tested, only 452 were found to recognize their target in HEK293 cell lysates[92]. Given that large caveat, the use of antibodies for staining (as with flow cytometry or CyTOF methods) is very different from their use in Western blotting or fluorescent sandwich immunoassays (SCBCs and microengraving), with each affording different quality checks. scWesterns, similar to standard immunoprecipitation-western methods, provide 2 separate measurements of each protein – the mass ladder (albeit of lower resolution than is common for bulk western blotting assays), plus a primary detection antibody [97]. However, absolute quantitation and absolute assessments of experimental uncertainty can be challenging. For multiplex fluorescent sandwich immunoassays, each individual protein assay provides two separate measurements per cell (since two antibodies per protein are used). Each individual assay can also be compared against every other assay in the panel for cross-reactivity[98], and each assay can be calibrated against solutions spiked with recombinant standards,[14, 98] thus providing assay readouts in terms of copy numbers per cell. However, on a cautionary note, recombinant standards may not be commercially available, or may be modified from the corresponding protein produced within the cells. SCBC platforms have an additional quality check in that individual protein levels can be assayed multiple times from the same single cell[14], thus providing a metric for experimental accuracy. Single cell methods that rely upon antibody staining of cells are the most challenging to quantitate, although experiments on FACS-sorted cells can provide validation that the antibodies used for staining surface markers are effective, thus providing a level of quantitation regarding the cell fractions that are positive or negative for specific markers.

Applications of single cell analysis: Uncovering New Biology

As tools have emerged that can analyze larger numbers of single cells with an increasing depth of analysis, a central emergent theme is that cellular biology is highly heterogeneous at virtually all molecular levels beyond the genome. Some of this heterogeneity is intrinsic to the nature of single cells (Box 1), while some of it is reflective of genetic or epigenetic influences [99, 100]. In many cases, it is becoming apparent that the heterogeneity is not arbitrary, and may be mined to yield a treasure trove of new biological information. A second emergent theme has been that a few cells can bias a population average.[101, 102]

Single cell genomic or transcriptomic analysis can permit lineage tracing of rare cell types (see below), which can provide insight into the origin (e.g. primary tumor or metastatic site)

of circulating tumor cells (CTCs), or into the use of CTCs as a liquid biopsy that reflects the originating lesion.[52, 62, 103, 104] A second application, pioneered by Quake's group, has been to provide a deep, molecular view of healthy[20] or diseased[105] tissue development via lineage tracing at the transcript and protein level. Related work has focused on identifying how multiple genetic defects associated with a single gene, but non-uniformly distributed throughout the tumor, influence tumor development and drug response.[53]

Advances in single cell proteomics have largely exploited the ability to interrogate combinations of secreted (or secrete-able) cytokines, chemokines and cytotoxic granules from highly defined cells of the hematopoietic lineage[106, 107]. This has permitted comparisons of the importance of immune cell function versus immune cell abundance[11, 13, 93, 98] (see below), and it has also revealed deeper insights into the hematopoietic lineage. Such studies are being applied in cancer immunotherapies, [29, 56, 102, 108], as discussed later. Single cell proteomics has also opened detailed characterizations of the structure of phosphoprotein signaling pathways.[14, 15] (see below and Box 1).

An emerging frontier is the use of microfluidics platforms that permit highly customized assays designed to correlate weak perturbations to single cells with changes in the transcriptome or proteome[42]. Two examples are studies that correlated cell motility with proteomic[90] or transcriptome analysis[32]. A third example was an analysis of how specific cancer cells respond to targeted inhibitors as the physical environment is altered from normoxia to hypoxia[31]. Other examples include studies of cellular responses to engineered molecular stimulations (i.e. periodic versus continuous),[109, 110] or studies designed to interrogate how one cell is influenced by another[111], including how that influence depends upon cell-cell separation distance.[28, 44] Such studies are enabled by the standardization of the relevant single cell assay biochemistries, and are limited only by the imagination of the researchers. They represent tremendously powerful approaches for decoding how genetic and epigenetic influences (such as drugs) are processed by living organisms.

Lineage tracing of Cellular Phenotypes

Single cell proteomics and transcriptomics may be used to understand the origins of cellular heterogeneity, as demonstrated by Dalerba and coworkers in colon cancer (Figure 3).[105] It was found that the transcriptional diversity of a human tumor could be largely explained by *in vivo* multilineage differentiation[98]. These findings are consistent with additional models[112] and mechanistic[113] investigations that demonstrate the ability of cancer cell differentiation (and de-differentiation) to maintain a phenotypic equilibrium within certain tumors. The study by Delarba and colleagues[98] was limited by the numbers of transcripts per cell, and the numbers of single cells, that could be analyzed a few years ago. The recent advent of high throughput, single cell global transcriptome analysis and exome sequencing should allow for such lineage tracing studies to dive significantly deeper into a host of developmental biology problems with relevance to both healthy and diseased states.

Cellular Functionality versus Cellular Abundance: Surprising properties of hematopoietic stem cells

Immune cells of the myeloid lineage are often considered the first responders of host defense against bacterial infection; meanwhile, hematopoietic stem and progenitor cells (HSPCs) have been thought to respond in a delayed fashion, so as to ensure sufficient production of myeloid cells consumed during an infection.[114-116] This response of HSPCs was considered to be a passive response to the depletion of downstream immune cells. Recent evidence suggests that HSPCs may participate directly by sensing systemically elevated cytokines as well as bacterial and viral components through cytokine receptors and Toll-like receptors (TLRs), respectively[117, 118]. Single cell functional proteomics (12-plex SCBC assays), combined with flow-cytometry cell sorting and genetically engineered mouse models, indicated that short term hematopoietic stem cells (ST-HSCs) and multipotent progenitor cells (MPPs) also have the capacity to respond to bacterial components via the TLR/necrosis nuclear factor kB (NF-kB) axis.[106] In fact, HSPCs were shown to be significantly more potent cytokine producers in terms of speed, breadth and especially quantity, than the conventional cytokine producers of the immune system, such as myeloid cells and lymphocytes[99]. Clustering of data from HSC SCBC assays revealed 4 functional subsets of LKS HSCs (defined as $Lin^{-}Sca1^{+}cKit^{+}$), secreting either a set of lymphoid or a set of myeloid-associated cytokines, or produced all proteins, or were completely silent[99]. The overall findings indicated that ST-HSCs and MPPs, although rare cells, can aggressively translate danger signals arising from an infection into the vigorous production of cytokine signals that allow them to directly self-regulate stress-induced hematopoiesis. These findings have multiple implications, with one possibility being related to patients who have undergone lymphodepletion regimens as part of a therapy procedure. Since the single cell functional proteomics assays are non-destructive to the cells, a logical next step in this type of work would be to analyze those functional subsets at the transcriptome level, to identify if there are specific cell surface markers that can be used to further differentiate those HSPC functional subsets.

High Throughput Drug Screening via Single Cell Phosphoproteomics

While single cell methods can provide a rich treasure trove of information, most are limited to analyzing only one to a few samples at a time. However, cellular barcoding techniques are evolving to remove this limitation.[22, 119] For mass cytometry, the basic idea is that cells are separated into a multi-well plate and barcoded with a unique combination of mass signatures that identify a given cell with its well location, and the experimental conditions (i.e. a specific dose of a specific drug) applied to that location. The cells are then analyzed all together, so that many experimental conditions are captured in parallel. For example, Bodenmiller and coworkers[108] used 7 mass-labeled barcodes to provide up to 2^7 barcoding capacity (128 possible addresses). This method was applied to a 96-well plate format to explore the kinetic and/or dosing influences of 27 inhibitors on 14 distinct peripheral blood mononuclear cell (PBMC) phenotypes (defined by 10 cell surface markers), via monitoring 14 phosphorylation sites per cell. From this data, IC50 values and percentage inhibition of the phosphorylation levels for all phosphorylated sites was extracted.

As described above, single cell, multiplex phosphoproteomic assays yield both the levels of the assayed proteins, as well as the protein-protein correlations. Thus, a major advantage and distinguishing feature of this high throughput screening approach is that it permits an analysis of how both on-target and off-target drug interactions influence the signaling networks, rather than just the relevant protein levels. A major challenge going forward will be to expand the multiplexing of these types of assays to capture more complete pictures of the phosphoprotein signaling networks, as well as additional networks associated with cellular proliferation, apoptosis, and metabolism.

Applications of single cell analysis in oncology

The single cell analytic methods discussed in this review are being applied towards addressing a number of fundamental biomedical problems, particularly in cancer biology and clinical oncology. Below, we discuss two such challenges that are deeply connected to modern drug discovery and development: cancer immunotherapy, and tumor heterogeneity.

Cancer Immunotherapy

The prototype model for our understanding of cellular differentiation and diversification in humans is the hematopoietic system. In fact, this knowledge has provided a scientific cornerstone behind the recent and remarkable advances in cancer immunotherapy.[120, 121] Single cell technologies have emerged as a critical set of tools for advancing this knowledge, often in dramatic fashion.

For cancer immunotherapies, single cell analytic tools are, or soon will be, providing critical guidance across multiple levels of biological information. Whether the immunotherapy is based upon dendritic cell vaccines[122], adoptive cell transfer[123] or checkpoint inhibitors[124-126], or some combination thereof, the primary tumor cell killers are T cells. Some of the most important biomarkers are the kinetic persistence and functional behaviors of specific anti-tumor T cell phenotypes across the course of a given patient's therapy regimen. For cell based therapies, the importance of designing clinical protocols that account for T cell differentiation has emerged as a key consideration.[127] In addition, patient-specific mutant epitopes[128] (called neoantigens) were suggested a few years ago to be a potentially important factor for understanding, or perhaps controlling, the anti-tumor specificity of an immunotherapy, and that suggestion has been borne out by recent findings.[108, 129-133] Closely associated factors are the T cell receptor (TCR) α/β chain sequences that recognize the specific expressed neoantigens with high avidity.

For much of this work, highly multiplex flow and mass cytometry methods[11, 13, 134, 135], and associated reagent development,[136-141] have provided the workhorse. These techniques allow a phenotypical characterization of immune cells, and also study intracellular signaling pathways. With the realization that T cell responses to cancer can lead to unprecedented levels of durable tumor responses in several cancers (melanoma, lung, bladder, lymphomas, leukemias), there is a need for further characterization of such responses that would lead to increased refinement in the therapeutic approaches and continued improvements in patient care.

To understand immune responses to cancer, it is of high interest to develop approaches that can match the TCR genes with their specific (or cognate) antigen, which is usually resulting from nonsynonymous somatic mutations specific for each cancer. [108, 136, 138-143] As each T cell has two TCR chains, it is important that they are defined from individual T cells to allow their correct pairing. Single cell analysis platforms coupled with DNA sequencing for TCR chains and paired neoantigens have the potential to revolutionize our knowledge about this critical interaction, guiding the success of cancer immunotherapy strategies.[21, 56] With the increased knowledge, it is easy to envision that in the near future, the definition of TCR chains that specifically recognize neoantigens in cancers may be translated into truly personalized cancer immunotherapy approaches for patients.

Once the recognition elements of T cells are fully defined, a next question is which T cell subsets are empowered to fight cancer. While these T cell subsets are each governed by specific transcription factors and can be identified by a series of surface molecules, [144] a specific subset can also exhibit a broad range of functional phenotypes, ranging from anti-tumor to immune-modulatory.[102] Single cell assays provide an unparalleled quantitative assessment of the different T cell subtypes, their progenitors, and their functional capabilities.[13, 16, 41, 102, 106] These assays are being applied to the characterization of T cell responses to cancer induced by several immunotherapy approaches[102, 106]. These methods are helping define how patients respond or resist to immunotherapy approaches, such as checkpoint blockade therapy, and may help guide the next generation of combination therapy studies that will be designed based on understanding what is lacking in patients whose immune systems do not respond to these therapies.

Advances in cell therapy manufacturing for adoptive cell transfer (ACT) approaches, where a large army of T cells are manufactured in the laboratory and re-infused back to patients, are being supported by new biotechnology approaches designed to guide higher level T cell characterizations.[29, 56, 108, 133] The knowledge on the TCR specificity and the generation of chimeric antigen receptors (CAR) to genetically re-direct T cell specificity to cancer, allows the manufacture of autologous cell therapies[145]. By applying highly multiplexed single cell analyses, the different T cell subsets can be surveyed before and after infusion of these cell therapies to patients and define which approaches lead to improved long term functionality to attack cancer. It has become clear that less mature cells that have long term repopulation ability (naïve, T stem cell and long term memory cells) are preferred in these ACT approaches[127], as more mature T effector cells have short term functionality and cancer may regrow after their infusion.[102, 146]

The next wave of advances in cancer immunotherapy will likely rely on the characterization of large numbers of single immune cells at the DNA, RNA and protein levels to de-convolute the complexity of immune responses to cancer and guide further therapeutic strategies. Lower order analyses fail to provide the necessary knowledge to understand immune responses to cancer and cannot explain the heterogeneity in patients response.

Understanding tumor heterogeneity

Intratumoural heterogeneity is increasingly recognized as a central hallmark of human cancer,[147] and describes three main types of variability: a) variation of mutational patterns

among tumors of the same histological type; b) variation of histological pattern within a tumor; and c) intratumoural mutational polyclonality, i.e. variation of the mutational complement within individual cells of a tumor.[148] In addition to mutational polyclonality, single cells within a tumor will intrinsically vary in the activity of their signaling[14, 15] and metabolic[27] networks, influencing the biological properties and therapeutic vulnerabilities of distinct tumor cell subpopulations. The impact on intratumoural mutational polyclonality and heterogeneity of signaling and biochemical networks on treatment and resistance are not currently well understood, in part because the standard genetic tools have not been well suited to measure it.

Tumors develop into a complex heterogeneous tumor mass, primarily through the intertwined forces of spontaneous somatic mutation coupled to clonal sequential selection for aggressive subclones.[147, 149-151] As tumors progress, new mutations are produced with an ever increasing frequency, accelerating the extent of intratumoural mutational polyclonality, and confounding treatment strategies.[148, 149] Intratumoural mutational polyclonality is enhanced in cancers that are associated with a causal environmental insult that directly damages DNA,[152] as well as by the progressive loss of key tumor suppressor proteins, and mutations in genes that control DNA damage sensing and repair [153].

The local microenvironment also provides a critical non-genetic force. Autocrine and paracrine interactions between inflammatory, stromal, endothelial and tumor cells are just a few factors that can influence the process of selection, and may yield cells bearing different mutations within different parts of a tumor or its distant metastases[154, 155]. Treatments also provide a source of non-genetic heterogeneity, expanding or collapsing tumour cell subpopulations depending on the treatment.[147, 148, 151]

Exome and genome-wide surveys have provided an atlas of driver mutations and a compelling road map for guiding the implementation of precision and personalized cancer medicine. However, intratumoural heterogeneity presents a serious challenge to this paradigm.[156] Fortunately, single cell technologies are poised to address this challenge.

Glioblastoma, GBM, the most common and lethal form of primary brain cancer, provides an illustrative example. GBM was one of the first cancers sequenced by The Cancer Genome Atlas.[157, 158] In that survey, 57% of tumors contained *EGFR* amplification and/or gain of function mutation, including *EGFRvIII*.[159, 160] *EGFRvIII* is oncogenic in mouse models when introduced in association with CDKN2A loss,[161] as commonly co-occurs in patients.[148] However EGFRvIII protein expression varies dramatically among the cells within a GBM and single cell DNA sequencing,[53] RNA sequencing,[21] as well as bulk analysis of DNA and RNA extracted from different regions of a tumor[162, 163] demonstrate considerable DNA, transcript and protein heterogeneity, including of EGFRvIII. Importantly, recent work suggests that the widespread variability of EGFRvIII gene, transcript and protein within individual cells of a GBM, may contribute to the resistance to EGFR-targeted therapies that is currently seen in the clinic.[113] In addition, single cell bar proteomics and metabolomics assays point to considerable variability in the signaling and metabolic networks of individual GBM cells[27] within an EGFRvIII+ tumor, potentially shedding new

light on mechanisms of resistance to targeted therapies, either pre-existing and/or adaptive that could be used to guide more effective combination treatments.

Challenges, limitations and outlook

The emergence of single cell ‘omics’-tools over the past half-decade has happened at a lightning pace, and the potential for their use in the discovery and development of broad classes of therapies and therapeutic strategies is very high. The resultant data sets do not just provide deeper views of biology that is already measured using existing methods, but also offer a fundamentally different view that is not masked by the intrinsic heterogeneity of a cell population. However, the newness of these single cell techniques also implies various limitations. For example, most methods discussed in this review have just recently emerged from academic labs, and so require significant skill sets and cross-disciplinary infrastructure that may be new to those in the drug discovery and development community. As cases in point, the three papers highlighted in the section “Uncovering New Biology via Single Cell Analysis,” have, on average, 13 authors representing 5 different departments or institutions. In other words, it can take a village to effectively integrate the technology, the biology, and the computational analysis. A second caveat is that algorithms for the in depth analysis of single cell data are even less mature than the experimental platforms, and effective visualization and interpretation of what are increasingly large data sets remains challenging, with techniques that vary across research groups. However, as methods mature, the experimental protocols, the reagents, and the computational analysis routines will become more standardized. This has, of course, largely happened for multicolor flow cytometry, and it is beginning to happen for CyTOF and single cell RNA-seq, but even those methods are still rapidly evolving, and they all involve dedicated user facilities.

Much of modern biological practice is designed around extracting correlations and associated statistical trends from biological systems that are intrinsically heterogeneous and thus noisy. The promise of single cell biology is to resolve and make sense of this confounding heterogeneity. Several studies highlighted in this review provide hints of the resultant clarity that can be achieved. It is likely that, as the tools of the field increase in terms of quantitation, throughput, and ease of use, the impact will be to fundamentally change the practice of biology, as well as the associated applied sciences, including drug discovery and development.

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Box 1

Biophysical Interpretation of Single Cell Data

The ability to quantitate the level of analytes from single cells provides fundamentally new insights into the cellular biology. For example, the abundance distribution of an analyte, as tabulated across many single cells, is also called the fluctuations of that analyte, and represents a unique single cell measurement. A certain width of the fluctuations is fundamental and unavoidable, and is reflective of the statistics associated with the many steps through which signals are processed by gene and protein networks. For purely stochastic[99] processes, the distribution width should narrow as the square root of the average copy numbers per cell of the analyte increases.[164] However, most analytes will not behave according to this limit and, in fact, the shapes of analyte distributions can reveal new biology, such as evidence of bistable steady states[165], or evidence that the cells are in a stable steady state,[42] or are unstable and responding to some perturbation (i.e. a drug). A relevant example of bistability might be a cell population that is comprised of both a quiescent state and an active state[166, 167], and thus yields differential responses to drugging.

Measurements of multiple analytes from the same single cells can be used to extract quantitative analyte-analyte correlations (and anti-correlations). Again, this is a uniquely single cell measurement. Consider, for example, the levels of the three hypothetical phosphoproteins (p-A, p-B, and p-C) shown in the figure. These proteins represent a small signaling network within a cell. Stimulation (or drugging) of the cell may collectively repress these phosphoprotein levels, as is reflected in the bulk immunoprecipitation assays. However, a more in depth picture of the signaling is revealed by an analysis of a statistical number of single cells, such as is presented in the two-dimensional scatter plots. Note that in the plots for the undrugged cells, all phosphoprotein levels are high, but only p-A and p-B are strongly correlated. Upon drugging, all phosphoproteins are repressed, but p-A and p-B are non-correlated, p-C and p-B are strongly correlated, and p-A and p-B are anti-correlated. This inferred correlation network is shown in the graphic, in which the protein levels are indicated by the sizes of the spheres, and the correlations are indicated by the edges. Correlation, of course, does not mean causation, but a correlation network generated at the single cell level can provide a rich set of testable hypotheses that may ultimately allow the chemical kinetic relationships that comprise a signaling network to be extracted. In principle, if one knows these relationships, then one can make accurate predictions regarding how a specific drug will disrupt the cellular signaling machinery. Improved measurement quantitation provides significant additional value. This is because most signaling cascades actually behave as excitable devices with built-in excitability thresholds, enabling them to integrate diverse temporal and spatial inputs to produce specific signaling responses.[168] In other words, the outputs of a signaling cascade are not typically linearly dependent upon the inputs, and quantitative assays permit such input/output relationships to be more accurately defined.

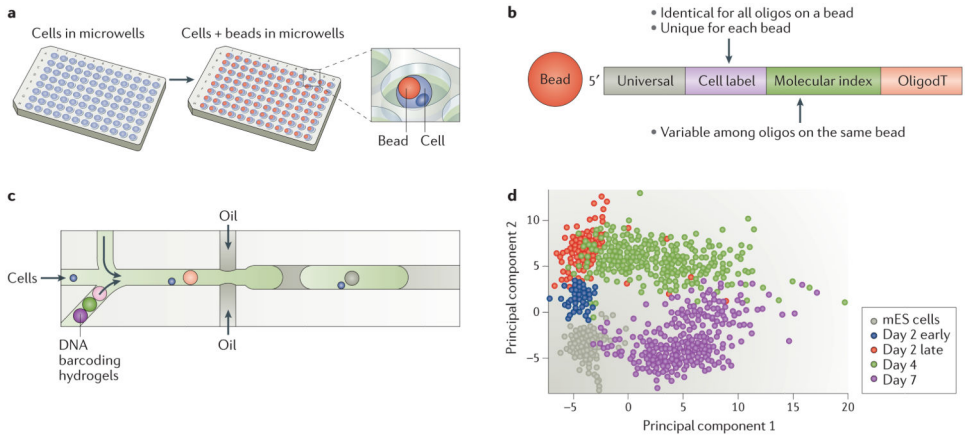

Figure 1. Quantitative single cell transcriptomic methods

Two separate, but conceptually similar methods, with similar throughput capabilities, are illustrated in this figure, along with representative data. **a.** The CytoSeq method is based upon isolating individual cells within 30 micrometer diameter (20 picoliter volume) wells, and then placing into each well a single barcoded bead. **b.** Each barcoded bead is designed with the shown structure. Each bead contains tens to hundreds of millions of distinct oligonucleotide primers which are each comprised of a barcode that identifies the bead (and thus the single cell), plus a molecular index (UMI) that is associated with a particular mRNA capture sequence. After bead and cell co-localization within a well, cells are lysed and mRNAs are captured via hybridization onto specific bead-bound oligonucleotides. The beads are then all removed from the well-plate, and all amplification reactions are carried out in a single tube. Adapted from [22]. **c.** The microdrop-based in Drop technique for single cell transcriptomics. For this method, single cells are entrained into a single droplet, along with a hydrogel microsphere. Each hydrogel microsphere contains photo-cleavable oligonucleotide primers that have a similar construction to the bead shown in part b, while the droplets contain the cell lysis buffers and reverse transcription (RT) reagents, so that the whole process from cell capture and lysis to signal amplification happens separately in each droplet. **d**. A snapshot of representative data from an inDrop study of the kinetics of differentiation of mouse embryonic stem (mES) cells following leukemia inhibitory factor (LIF) withdrawal. For this plot, data sets representing 5 time points are analyzed using principal component anaysis to reveal asynchrony in mES cell differentiation. Each dot represents a single cell. Adapted from ref [71].

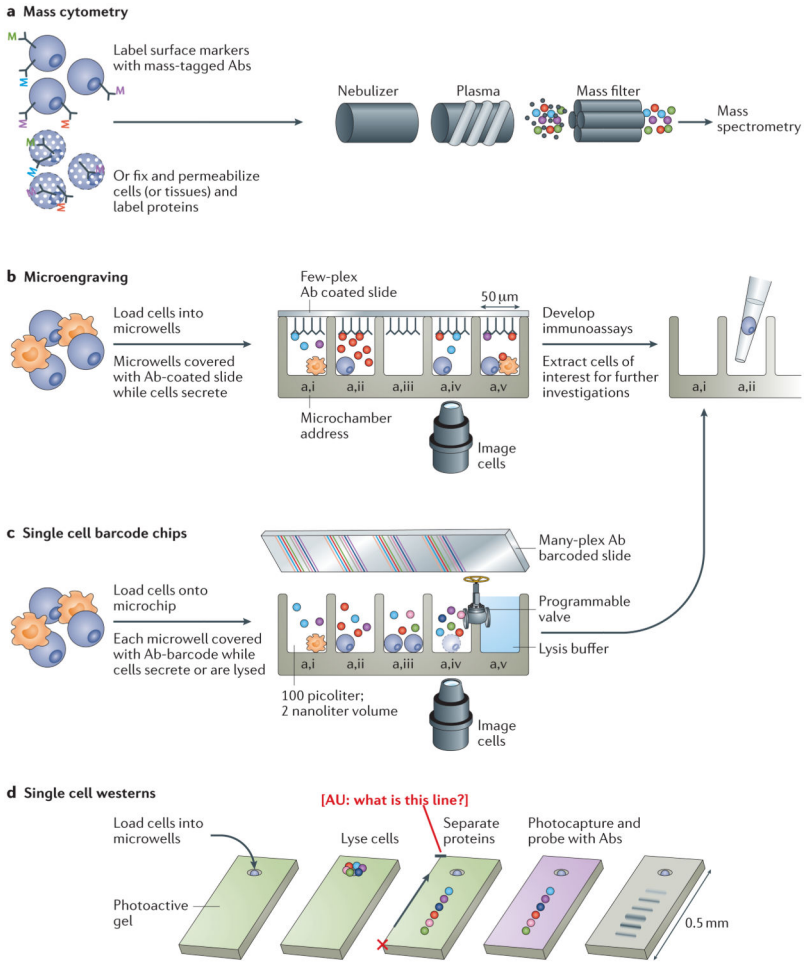

Figure 2. Emerging single cell proteomics methods

a. Mass cytometry uses antibodies (Abs), encoded with transition metal containing mass tags, to label proteins of interest. Cells are fixed and permeabilized so as to permit Ab-staining of cytoplasmic proteins. Single cells are entrained into vapor and atomized. A mass filter separates the transition metal atoms, which are then mass analyzed. The abundance and identities of the transition metal atoms are traced back to the Ab staining reagents. **b.** The microengraving technique utilizes a microchip with many thousands of microwells, into which between 0 and a few cells of interest are loaded. An Ab coated coverslide is placed over the microchip to capture specific secreted proteins. Microchip addresses are correlated with regions on the coverslide and with microscopy images to associate a given cell with a given secretion profile. Captured proteins are detected using fluorescent secondary Abs, with different proteins identified using different fluorophores. The coverslide can be replaced during the time-course of an experiment to capture single cell secretion kinetics. Cells of interest may be removed for further analysis. **c.** Single cell barcode chips (SCBCs) contain up to a few thousand microchambers, into which between 0 and a few cells are loaded. An Ab-barcoded glass slide is patterned so that each microchamber contains a complete, miniaturized Ab array onto which secreted, or following cell lysis, cytoplasmic or membrane proteins are captured. Protein assays are developed using fluorescently-labeled secondary

Abs, with different proteins identified according to the spatial location of the immunoassay within the barcode. If cells are not lysed (only secreted proteins detected), then the cells remain viable and may be further investigated. **d.** Single cell Westerns are miniaturized variants of traditional Western Blotting methods, with $\sim 10^3$ single cells analyzed per microchip.

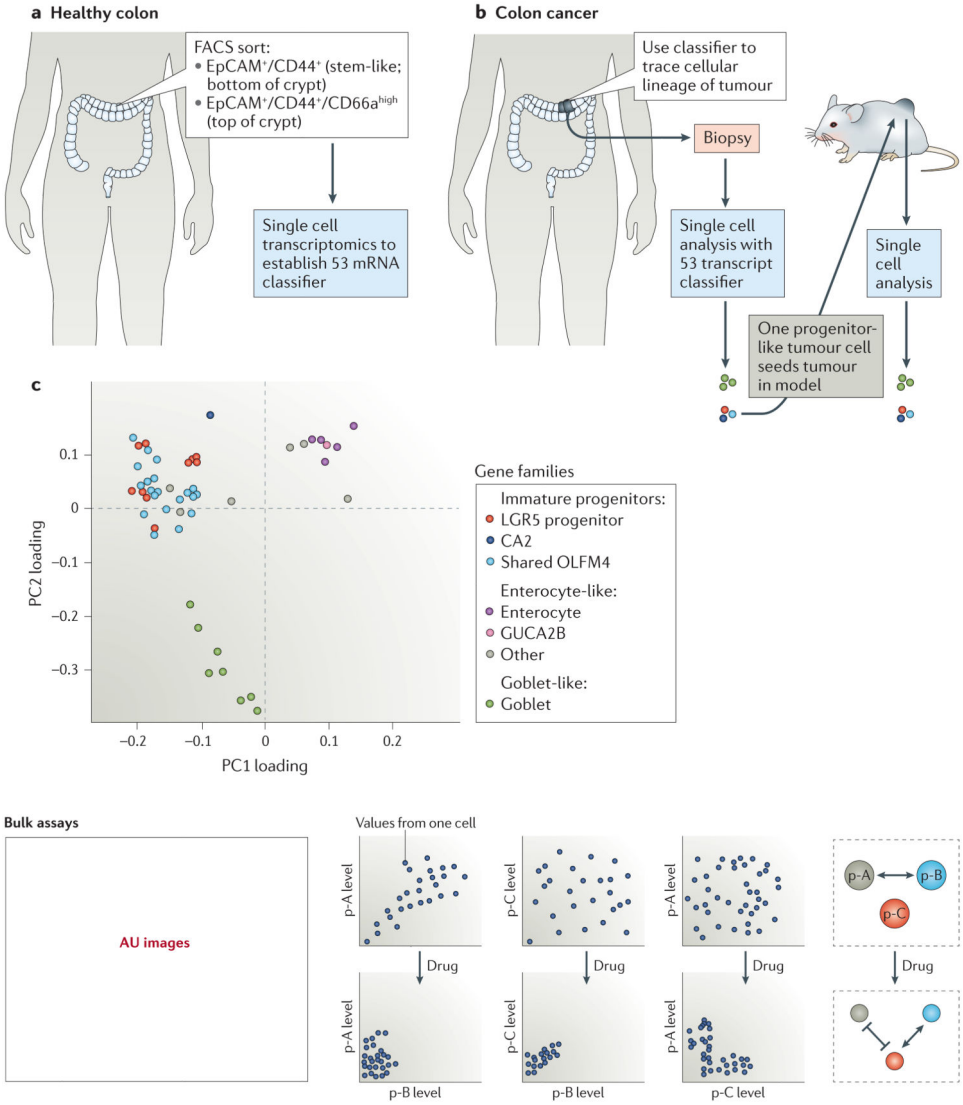

Figure 3. Single cell analysis traces the lineage of a colon cancer

The work flow proceeds from left. A biopsy of a healthy colon is analyzed using FACS to separate cells extracted from the crypt-like structures of the colon epithelium. The bottom regions of the crypts are enriched in stem cell-like populations, with those cells identified as $EpCAM^{+}/CD44^{+}$. More differentiated enterocyte and goblet cells are found near the top of the crypts, and are defined as $EpCAM^{+}/CD44^{-}/CD66a^{high}$. Single cell, multiplex transcriptomics is used to develop a 53 gene expression classifier. Principal component analysis (PCA) of the single cell data resolves the major cellular subpopulations. The genes that define those subpopulations are plotted with respect to how they are represented within the two dominant principal components. The plot reveals how the classifier resolves immature progenitors (top left of graph), enterocyte-like cells (top right), and goblet-like cells (bottom left). Classifers of these populations, also identified from hierarchical clustering of the single cells transcriptome data, provide the color coding for each mRNA on the plot. Once established, the classifier was used to analyze cells collected from a patient colon cancer tumor, and to show that the tumor cells (drawn with a red border) are largely

goblet-like and immature progenitors. A single immature progenitor tumor cell is sorted from the patient tumor using FACS, and implanted into a mouse model to grow a monoclonal tumor. Analysis of that tumor reveals a cellular composition reminiscient of the original patient tumor, implying that the tumor cellular heterogeneity can originate from expansion and lineage differentiation of a single progenitor-like cell. The principal component plot is adapted from reference [105].

Table 1
Characteristics and Capabilities of Single Cell Proteomics Methods

Single Cell Proteomics Method	Protein Detection Method	Comments	literature
Fluorescence Activated Cell Sorting (FACS)	Staining with fluorophore-labeled antibodies	• Standard for cell sorting based on membrane protein cell surface markers • High throughput tool with excellent statistics • Mature technique. • Multiplexing is colorimetric. • Typically requires large sample size; • Sorted and analyzed cells are viable and for subsequent analysis. • Commercial product (many vendors).	References: [8, 11]
Mass Cytometry (CyTOF)	Staining fixed cells with mass-tag labeled antibodies	• Good for cytoplasmic proteins; • Excellent statistics; • Demonstrated as a drug screening tool, • >30 proteins assayed per cell. • Multiplexing is via mass spectrometry. • Applicable to fixed tissue analysis. • Commercial product (Fluidigm). References	References [13, 15, 39, 45, 108]
Single Cell Barcode Chips (SCBCs)	Spatially-encoded antibody array for fluorescent immuno-assays of secreted proteins or analytes released from lysed cells	• Permits absolute quantitation. • Small (10^2-10^3 cells) biospecimen size ok. • Demonstration of >40 proteins assayed per cell. • Secreted proteins detected from viable cells. • Some designs integrate cell lysis to permit cytoplasmic protein assays and integrated protein/ metabolite assays. • Analysis of cell-cell interactions. • Small (10^3-10^4 cells) biospecimen size ok. • Cost effective. • Multiplexing is via spatially encoded arrays. • Commercial Service (Isoplexis).	References [12, 14, 27, 93, 95]
MicroEngraving	fluorescent immunoassays of secreted proteins	• Small numbers of secreted proteins.	References: [35, 80, 89, 103]

Single Cell Proteomics Method	Protein Detection Method	Comments	literature
		• >10^4 single cells assayed in parallel. • Cost effective. • Permits kinetic studies of protein secretion • recovery of analyzed cells for further analysis • analysis of cell-cell interactions[103]. • Small (10^2-10^3 cells) biospecimen size ok. • Cost effective. • Multiplexing is colorimetric.	
Single Cell Western Blotting (scWestern)	Miniaturized, automated Western Blotting on a microchip	• Small (10^2-10^3 cells) biospecimen size ok. • 10^3 cells assayed per microchip • multiplexing to ∼12 proteins demonstrated • permits cytoplasmic proteins from lysed cells; • reasonably fast (4 hours). • Provides protein ladder reference. • Relative quantitation.	References [81]