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Journal of Quantitative Cell Science PART A

Genomic Cytometry and New Modalities for Deep Single-Cell Interrogation

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Received 6 November 2019; Revised 28 June 2020; Accepted 7 August 2020

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Published online in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.24209

Abstract

In the past few years, the rapid development of single-cell analysis techniques has allowed for increasingly in-depth analysis of DNA, RNA, protein, and epigenetic states, at the level of the individual cell. This unprecedented characterization ability has been enabled through the combination of cytometry, microfluidics, genomics, and informatics. Although traditionally discrete, when properly integrated, these fields create the synergistic field of Genomic Cytometry. In this review, we look at the individual methods that together gave rise to the broad field of Genomic Cytometry. We further outline the basic concepts that drive the field and provide a framework to understand this increasingly complex, technology-intensive space. Thus, we introduce Genomic Cytometry as an emerging field and propose that synergistic rationalization of disparate modalities of cytometry, microfluidics, genomics, and informatics under one banner will enable massive leaps forward in the understanding of complex biology.

Key terms

genomic cytometry; technology; cytometry; genomicsmicrofluidics; single-cell

THE cell is the basic unit of life and is capable of a vast array of biological complexity. In order to understand how different populations of cells can functionally coexist to form organs, organisms, and indeed disease, it is critical to profile all aspects of the individual cells. The capability to perform in-depth single-cell analysis has provided us with a more complete understanding of disease, development, and normal function. Moreover, the application of single-cell genomic technologies has already identified many of the molecular features of cell populations within tissues, organs, and diseases.

Techniques that together comprise the field of Genomic Cytometry have already been used to reveal a fundamental aspect of biology. Most notably, that cell populations are more heterogeneous than ever imagined. Each individual cell is unique in terms of space (e.g., physical position in tissues and/or organs), time (e.g., phases of cell cycle, activation or developmental state), and molecular profile. This uniqueness makes understanding the underlying biology a significant challenge.

While in the past, scientists could interrogate, enumerate, and classify cell types according to their appearance under the microscope, this analysis is limited in the number of characteristics that can be simultaneously probed, the rate at which observations can be made has relied heavily on the individual interpreting the data. Modern flow cytometry emerged to give an additional level of detail to the classification process. By making use of multi-parameter, multi-laser instruments, flow cytometry has redefined cell classification at the molecular level, aided the discovery and definition of major and minor cell subsets, and has quickly become an essential tool for dissecting the functional complexity of cell populations. In general, however, it is primarily used to identify cellular protein expression profiles and despite being able

to process many millions of cells in a rapid manner, it is also hampered by limited dimensionality and the inherent loss of anatomical context.

Limits around fluorochrome uniqueness and detector numbers result in characterization ability topping out around the 30-parameter mark. In line with advances in fluorescent cytometry and the development of spectral cytometers (1), instruments such as the CYTOF (2) have matured and are now capable of 40+ parameters (3, 4). While there is some debate around the benefits and trade-offs associated with the use of mass cytometry (5), the advent of scanning ablation and ion beam systems (6–8) has helped to bridge the gap between imaging and flow cytometry. In doing so, they have provided tools that allow 2D reconstruction of tissue sections such that anatomical location of protein expression can be performed down to the micrometer range. A recent study by Keren et al. has improved this resolution down to 260 nm (9). These imaging systems, however, tend to be much slower than traditional cytometry and have their own unique challenges.

Given that even the most advanced methods in fluorescence and mass cytometry are still limited, it is clear that new methods must emerge to allow deep single-cell characterization. In order to be widely applicable in biological studies, these systems should provide throughputs similar to current fluorescent flow cytometric techniques while also providing improved dimensionality (hundreds to thousands of parameters simultaneously). By combining advances in cytometry with the tools emerging from the field of single-cell genomics, we are entering a new era of Genomic Cytometry. With the tools and workflows being created by today's emerging genomic cytometrists, we now can understand, in a concerted manner, many aspects of individual cells.

Genomic Cytometry techniques, while focused on the single cell, allow us to identify and characterize a group of single cells that share a similar function. The characteristics able to be probed are no longer limited to protein expression profiles, but now include aspects such as DNA, RNA, proteins, metabolites, and even epigenetic modifications. This unprecedented ability to sensitively interrogate large numbers of individual cells at a reduced cost is accelerating discovery and challenging existing paradigms in cytometry. Perhaps more importantly, this technological leap is transforming how we understand basic and translational biology.

The single-cell multi-omics revolution has fostered a parallel development of computational approaches, necessary to integrate and understand the data generated from single-cell genomic techniques. These methodologies and approaches have been described elsewhere (10–14). In this review, we analyze the factors and motivations that have given rise to the field of Genomic Cytometry. We also provide an overview of the tools currently available in this space.

Unraveling Cellular Complexity

Cells are complex assemblies of macromolecules and chemicals that function as a single unit during homeostasis, development, and disease. Currently, there are a multitude of different tools and methodologies that can be used to characterize a cell. These tools can measure the physical characteristics of a cell (such as size, deformability, electrical impedance, and density) as well as biochemical aspects such as DNA, RNA, and protein (concentration, monomer composition, and chemical status including mutation, acetylation, phosphorylation, methylation, etc.). Importantly, emerging tools are increasingly allowing the simultaneous characterization of these parameters. This is known as multi-omics.

Although many aspects of a cell are able to be assessed by traditional cytometry, it has primarily been leveraged to characterize protein expression profiles at the level of the individual cell. From the early advent of fluorescence cytometry in the late 1960s (15) and cell sorting in 1965 (16), the underlying technology has remained relatively static. Instrument manufacturers have added additional laser lines and increased detector numbers in order to improve multiplexed single-cell characterization; however, flow cytometry is still hampered by a lack of spectrally unique fluorochromes. Recent developments in dye technology, particularly around tunable polymer-based dyes (17), have allowed flow cytometry assays to reach into the 28 color range (18–22). However, if we look at the total cellular complexity, it is clear that even high dimensional fluorescent flow cytometry is incapable of completely characterizing the full range of cellular identities and cellular states.

To understand the challenge of fully characterizing a single cell, we must look at the complexity within cells (Table 1). The human genome is composed of 3 billion nitrogenous bases. These are structurally organized into regions that can be transcribed to RNA and subsequently translated to protein. These regions are known as genes. Although there is still conjecture around the number of genes (24, 25), studies suggest that the number of human genes sits somewhere in excess of 19,000 (26–29). Of the estimated 19,000 protein-coding genes, it is possible to make many different proteins, some authors suggest as many as 100 different proteins can be made from

Table 1. Potential complexity of the individual human cell

MEASURABLE CHARACTERISTIC	ESTIMATED OBSERVABLE NUMBERS
DNA	3,000,000,000 (bases)
Epigenetic states	
Open chromatin regions (enhancers and promoters)	100,000–150,000 (peaks)
DNA methylation	25,000 (CpG islands)
Three-dimensional genome architecture	~7,000,000 (long-range contacts) (23)
RNA	19,000 (coding genes)–100,000 (noncoding RNAs)
Proteins	>19,000
CD markers	>400

each gene (30). To date, the Human Cell Differentiation Molecule (HCDM) group has defined over 400 cluster of differentiation markers (31).

In addition to regions that code for proteins, noncoding regions of the DNA also exist. These regions include enhancers, insulators, and promoters, which are key for gene expression regulation and thus important markers of cell-type. Epigenetic mechanisms like DNA methylation, histone post-translational modifications, expression of noncoding RNAs, three-dimensional, structure and nucleosome positioning all shape the conformation of the chromatin to regulate gene transcription adding an additional layer of complexity to the characteristics of the cell (32).

Common Genomic Cytometry Approaches

Broadly speaking, it is possible to arrange Genomic Cytometry techniques into five main methodology categories. These categories are shown in Figure 1, they are:

1. Plate-based approaches (making use of traditional Fluorescent Activated Cell Sorting [FACS]).
2. Microfluidics: (1) Droplet-based microfluidics (aqueous reaction chambers created within an oil-in-water droplet); and (2) solid microfluidics (miniaturized single-cell handling tools with associated molecular workflows for downstream characterization).
3. In situ combinatorial indexing (using the cell as the reaction chamber itself).
4. Image-based approaches (making use of direct imaging or spatially traceable barcodes to create high dimensional, anatomically relevant images).

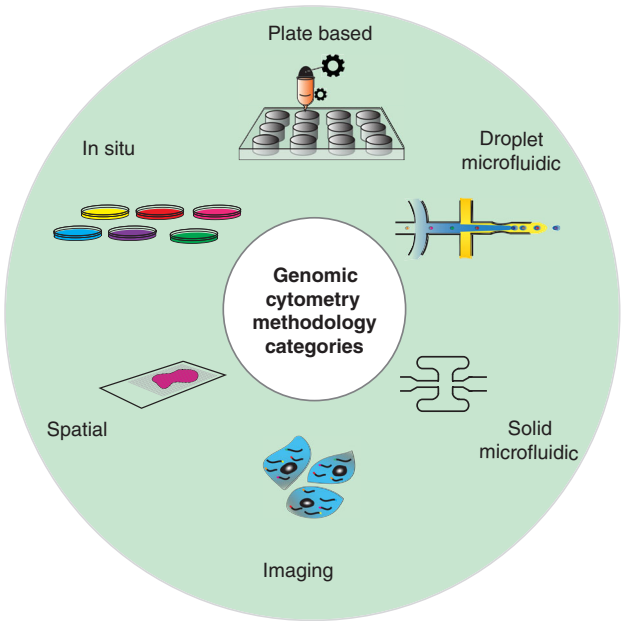

Figure 1. The main methodology categories that comprise the field of Genomic Cytometry.

5. Spatial transcriptomics (combining basic imaging with novel positionally traceable cellular barcodes).

Plate-Based Approaches

Plate-based assays are the most familiar to the traditional cytometrist and are one of the few high throughput Genomic Cytometry methods that currently allow active single-cell deposition. Active cell deposition is usually achieved using FACS, which allows selective deposition of cells based on characteristics measurable by traditional flow cytometry techniques.

Mechanically, plate sorting is most commonly achieved through the use of electrostatic droplet-based cell sorting. In these systems, single cells are sequentially flown through an interrogation point, characterized and deflected into the well of a microtiter plate. By incorporating a system capable of moving the microtiter plate with repeated micron-level accuracy, it is possible to target individual wells sequentially. Cells are deposited into 96- or 384-well microtiter plates; however, in some cases higher density plates can be used. In addition to ensuring the target cell is deposited into the correct well, most instruments will allow the operator to control the likelihood that (1) a cell is in the deflected drop, (2) more than one target cell is not deposited, and (3) the nontarget cell contamination is minimized. For traditional FACS, these are controlled through the application of a sort mask and allow the operator to balance cellular throughput and deflection accuracy with the requirements of high-speed cell sorting.

As current cytometers have not yet overcome the randomness of cell arrival times, many cells that meet the selection criteria are not deposited into the sort well. Single-cell masks look at the predicted position of the cell in the individual drop and will abort the sort if the cell is located in either the leading or trailing edge of the drop. This means that single cells on the periphery of the drop are not deflected and adds to the cell losses associated with the requirement to abort sort packets that contain coincident events. The ability to deterministically control cell location with relation to time and space will remove inefficiencies associated with the Poisson distribution of cells in drops and will result in higher throughput, lower loss single-cell approaches, while still retaining the characterization complexity afforded by traditional FACS.

While electrostatic droplet-based FACS is by far the most common method for depositing cells into microtiter plates, emerging technologies such as the CellenONE and the WOLF cell sorters are providing alternatives. Both of these systems use a low-pressure microfluidics-based approach and can thus be used on highly friable cell types that may be sensitive to the stresses of traditional FACS. The CellenONE system is a unique ultra-low volume liquid handler that utilizes an active image-based cell sorting approach to improve cell deposition accuracy while simultaneously minimizing cell loss (sort aborts are simply collected without dilution for subsequent reanalysis and deposition). Both the WOLF and the CellenONE systems are slow when compared to FACS, and can only handle limited cell numbers, for this reason, they

tend to have specific applications and often require pre-enrichment steps when dealing with rare cell populations.

Modern FACS instruments also include a software module that tracks the characteristics of the cell sorted and links this to the well coordinates. This process, known as index sorting, is critical to multi-omic studies as it allows protein expression profiles (captured as part of the sort decision) to be cross-correlated to the genomic data generated in downstream assays.

Assays that take advantage of a plate-based approach include: Smart-Seq (33), Smart-Seq2 (34), Smart-Seq3 (35), STRT-seq (36), STRT-seq-2i (37), Cell-Seq, Cell-Seq2 (38), MARS-Seq (39), mcSCRB-seq (40), Qartz-seq (41), Qartz-seq2 (42), scBS-seq (43), and single-cell HiC (44).

Microfluidics

Microfluidics have expanded massively in popularity in the past two decades (45). In recent years, the field has also made a significant contribution to both our understanding of biology and to many areas of health care (45–48). Using microfluidics, entirely new assays can be created and traditional assays miniaturized. With reactions performed in the nano to pico-liter range (49), microfluidic-driven miniaturization can result in log fold difference in the reaction volume. Because miniaturization can improve reaction efficiencies by simultaneously reducing reagent and sample input, microfluidics is becoming increasingly critical to our ability to perform high-throughput, high-resolution, high-sensitive assays in a cost-effective manner.

Microfluidics is used in a range of technologies but with reference to genomics, its application in massively parallel sequencing technologies was a significant contributor to the precipitous drop in sequencing cost. It is also being used in most of today's commercially available, high-throughput Genomic Cytometry platforms, such as the 10× Genomics Chromium and BD Rhapsody, Dolomite Bio Nadia, Missionbio Tapestri, ICell8, Biorad ddseq, InDrops, and Fluidgm C1 systems. To assist with the categorization of the many microfluidic approaches available, we have split the techniques into two subcategories, those that involve droplets and those that utilize miniaturized solid reaction chambers.

Droplet Microfluidics

The realization that droplet microfluidics is useful in the study of biology came of age with the simultaneous publication of two seminal papers out of Harvard and the Broad Institutes in 2015 (50, 51). These papers showed, for the first time, the application of high-throughput droplet-based generators in single-cell RNA-seq (scRNA-seq). Since then, commercial systems such as the 10x Genomics Chromium, Biorad ddseq, Dolomite Bio Nadia, and the Missionbio Tapestri systems have been released. Among these, the 10× Genomics Chromium system has the broadest acceptance. This is likely due to the fact that it was the first to include a highly defined kit-based approach combined with an accessible data interface. At the time, this created a uniquely user-friendly ecosystem. With this, a biologist without deep expertise in microfluidics and genomics could generate single-cell data with relative ease. As the field becomes more mature and competitors increasingly enter the market, we expect the dominance of a single platform to be significantly challenged.

Mechanistically, droplet-based microfluidic systems work by mixing two immiscible liquids to create a water-in-oil emulsion. The oil forms a self-contained reaction vessel around an aqueous phase. The aqueous phase contains both cells and a bead containing uniquely barcoded mRNA capture probes in lysis buffer. For 3′ scRNA-seq assays, the capture probe contains a poly dT region of around 22–25 nucleotides, which binds to polyadenylated transcripts released upon cell lysis. Thus, as the mRNA is released the polyadenylated region of the transcript is immediately bound to an oligo containing a (1) a cell barcode, (2) a Unique Molecular Identifier (UMI), and (3) a nucleotide region that assists with subsequent transcript amplification. As the aim of these systems is to co-locate a single bead with a single cell to a single droplet, the RNA profile for each captured cell can be obtained by informatically pooling of cell barcodes. The UMI tracks individual transcripts allowing for correction of amplification bias. The utilization of the dual barcode approach allows digital transcript counting at the level of the single cell.

Droplet volume is dependent on the flow rates and the chip geometry and while this can be used to create a wide range of droplet sizes, the droplets used in scRNA-seq applications generally range from a few hundred pico-liters to a few nano-liters (52). Droplet volume has been shown to be inversely related to the number of transcripts detected in the final library, for this reason, applications such as DroNC-Seq (designed for polyadenylated RNA transcripts from the cell nucleus) are better suited to systems that produce smaller droplets (75 vs 120 μm diameter droplets) (53).

Droplet microfluidics have been extensively utilized in the context of Genomic Cytometry. In addition to the work mentioned above, it has been used to profile transcriptomes at single-cell resolution (54), and other non–RNA-based applications. These include, (1) single-cell epigenetic approaches such as: single-cell ChIP-seq (55, 56), dscATAC-seq (57, 58) ChIA-Drop (59) single-cell ATAC seq (23); and (2) single-cell DNA approaches like single-cell gDNA-seq (60–62) and a variety of multi-omic workflows including CITE-Seq (63), REAP-seq (64) (protein and transcriptome), ECCITE-seq (65) (transcriptome, protein, clonotypes, and CRISPR perturbations), and SNARE-seq (66) (chromatin and transcriptome).

Solid Microfluidics

Solid microfluidic platforms use physical barriers to create individual reaction chambers, often at high physical densities but always with ultra-low volumes. These chambers can be made from a variety of materials but commonly include plastic, metal or polydimethylsiloxane (PDMS). Because solid microfluidics uses a physical confinement on solid substrates, it is possible to perform imaging on the cells in the well. If each well location can be associated with the unique cell barcode, then it is also possible to associate this data with the

downstream genomic characterization. Systems that allow this tend to have lower throughput and include the Fluidgm C1™ and ICell8™ systems.

The Fluidgm C1™ system is perhaps the best know solid microfluidics platform. The C1 utilizes an intricate microfluidics architecture to provide high-level control of the complex molecular reactions required for single-cell analysis. The C1 system has been used in a number of studies characterizing single cells at the level of RNA, DNA, and epigenetic changes (67–69). Despite the systems advanced approach, problems have been identified and care should be taken with its use (70). The ICell8™ system is a commercially miniaturized plate-based system that allows high-density fluid handling to achieve microfluidic scale single-cell genomics.

Recently, Becton Dickinson has released a high throughput scRNA-seq system, the BD Rhapsody. It uses a similar approach to the CytoSeq (71) and Seqwell protocols (72). By using a microwell approach, the Rhapsody system can place a single bead in virtually every well and does not expose the cells to the same pressures associated with droplet generation. This may be an important consideration when working with cells highly sensitive to pressure-related stress.

In addition to high recovery rates, Rhapsody workflows also allow both a whole transcriptome as well as a targeted transcriptomics approach. While commercial modifications have occurred, the molecular workflow is similar to that used in many droplet-based scRNA-seq techniques. The targeted scRNA-seq approach, however, is currently unique to the Rhapsody and while it requires a priori knowledge of the system being interrogated, it allows transcripts of interest to be deeply probed without incurring the high sequencing cost associated with reading common housekeeping and lowly informative transcripts. Depending on the panel, it is possible to obtain the same sequencing saturation, with up to 10 times less sequencing reads than that obtained when using a WTA approach (73).

In Situ Combinatorial Indexing

In situ single-cell methods provide an ingenious way to use the inherent structure of the cell or nuclei as the reaction chamber itself. This is achieved by first fixing the cell using methanol, or beginning with an intact nucleus, and subjecting these to multiple sequential barcoding steps using a split-pool approach. Through successive integration of molecular barcodes into the cell/nucleus itself, in situ combinatorial methods are capable of building up a library of uniquely barcoded single cells. For these methods to work effectively, it is critical to ensure that the number of barcodes that can be created is well in excess of the number of cells/nuclei being labeled. As the total number of barcodes possible is a combination of (1) the number of unique starting oligos and (2) the number of successive split-barcode-pool-split steps, these methods require careful balancing of cell inputs to available barcodes. Failure to do this will result in cells/nuclei sharing the same barcode.

Notable examples of in situ combinatorial approaches include, a 2015 method to perform single-cell combinatorial indexing ATAC seq (74), sci-RNA-seq (single-cell combinatorial indexing RNA sequencing) (75), split-pool ligation-based transcriptome sequencing (SPLiT-seq) (76), single-cell combinatorial indexed sequencing (SCI-seq) (77), sci-CAR (78) single-cell transposome hypersensitive sites sequencing (THS-seq) (79), single-cell DNA methylation (sci-MET) (80), droplet-based sci-ATAC (57), and single-cell Hi-C (Sci-Hi-C) (81). Recently, SplitBio announced commercial release of a single-cell RNA sequencing kit utilizing in situ combinatorial indexing.

Image-Based Approaches

In contrast to spatial transcriptomic systems that rely primarily on spatially attributable cell barcodes, image-based Genomic Cytometry techniques rely on in situ imaging of cells. These systems have been used to directly image the location of both RNA and protein in tissue sections. Because sample handling is reduced and solid tissue does not require digestion, such systems may provide the most representative method to study cellular composition in solid tissues. Example systems include the Codex and a number of highly multiplexed fluorescent in situ hybridization (FISH)-based approaches.

Highly multiplexed FISH approaches take advantage of specially designed probes combined with multiple rounds of hybridization and imagining to build anatomically localized transcript maps on tissue sections. Examples of such approaches include MERFISH (82), STAR-map (83), Seq-Fish + (84), or DNA microscopy (84).

The Codex system (85) can perform high-dimensional image-based protein detection with the use of oligo-conjugated antibodies. The system has been adapted for both slide imaging, super-resolution imaging, and has also been shown to work with volumetric imaging. By using a series of fluorescently labeled bases and relying on the specificity of complementary binding of fluorescently labeled base pair sequence to the oligo attached to the antibody, it is possible to perform highly multiplexed protein detection in tissue. Codex has been validated in both FFPE and frozen samples and can detect more than 40 proteins from the same individual cell.

Spatial Transcriptomics

Spatial transcriptomic workflows are complicated and require complex bioinformatics pathways. However, they can be simplified to a number of key steps, (1) a tissue section is cut, (2) section is laid on a solid imageable surface containing immobilized region-specific capture probes (these are akin to the cellular barcode used in other methods), (3) the section is imaged, (4) the sample is then permeabilized, and finally (5) the polyadenylated mRNA is captured by spatial probes. Following this, cDNA is synthesized, libraries are created, and then sequenced. As the location of the unique oligo sequence for the capture probe can be traced back to a discrete physical location, it is possible to create a single-cell transcriptomic library that retains anatomical information. The resolution of the system is governed by both the spot size of the deposited

capture probes and the distance between the centers of adjacent capture probe spots. The very first spatial transcriptomic system (86) had a spot size of 100 μm, with a distance between spot centers of 200 μm, and an estimated 200 million capture oligos per spot. Academic systems with spot sizes approaching that of the single-cell include Slide-seq (87) and HDST (88). These systems have a resolution of 10 and 2 μm, respectively. Recently, alterations to the molecular component, including "the bead barcode synthesis, array sequencing pipeline and the enzymatic processing of cDNA" of the Slide-seq method, were used to improve sensitivity by an order of magnitude (Slide-seqV2) and allow better transcript representation (89).

Commercial methods such as the Visium from 10× Genomics are currently available but not yet in widespread use. These methods are also not yet at the level of the single cell. Instead, they have spot sizes that contain many cells and have large gaps between the spots. The Visium platform uses spot sizes of 55 μm, with the separation between spot centers being 100 μm. One caveat of systems like this is the need of permeabilization time optimization which will vary from sample to sample. We expect that as spatial transcriptomics are further developed, they will become a valuable method for deeply characterizing patient disease. However, until standardized protocols across a number of tissue types can be determined, the widespread clinic adoption of such systems will likely be hindered.

Multi-Omics

Multi-omics is the science of combining measurements afforded by the different omics modalities on the same sample. In Genomic Cytometry, multi-omics involves the measurement of more than one class of cellular characteristics at the level of the single cell simultaneously. Generally, this includes the measurement of (1) RNA with protein, (2) RNA with DNA, (3) DNA with protein, or (4) epigenetics analysis with protein. However, approaches allowing three modalities to be probed simultaneously are emerging.

Low throughput multi-omics has been possible since the advent of FACS-based index sorting for downstream scRNA-seq applications. By simply varying the downstream genomic analysis method, it is possible to use index sorting for a multitude of single-cell multi-omic studies. This approach is often used in mid throughput scRNA-seq plate-based assays such as Smart-Seq (33, 34), Cell-Seq2 (38), and MARS-seq (39). Even inherently multi-omics methods such as G&T-seq (90) can be combined with index sorting to add a protein dimension to the multi-omic analysis. The idea of using index sorting to boost multi-omics identification of single-cell at the level of RNA, DNA, and protein has recently been leveraged in the TARGET-Seq (91) protocol.

In order to facilitate high-throughput multi-omic approaches involving protein detection, a number of oligonucleotide-conjugated antibody techniques have been developed. These include CITE-seq (63), REAP-seq (64), and Ab-seq (92). The use of oligonucleotide labeled antibodies has allowed a substantial step forward in the ability to perform high dimensional single-cell protein detection. By incorporating a unique oligo onto the antibody, it is possible to detect the extracellular protein expression on the cell using common 3′ scRNA-seq. The oligos attached to the antibodies contain (1) an antibody specific base pair sequence (to identify antigen specificity), (2) a PCR handle (to allow amplification during library preparation), and (3) a poly-A sequence (to allow the antibody conjugated oligo to be captured by the polyT region of the capture probe). This approach has been commercialized by both Biolegend and Becton Dickinson.

The use of oligonucleotide-conjugated antibodies has been shown to be effective at detecting many antigens. However, the technology is relatively new, and care should still be taken when designing panels. While we do not yet have guidelines for panel design, factors such as (1) epitope expression density, (2) cell numbers stained, (3) sequencing depth, (4) relative expression ratios, and (5) library complexity are likely to affect the outcome of oligo antibody characterization studies.

One of the criticisms of oligonucleotide-conjugated antibodies is that the sequence allocation required to detect all bound antibodies is dependent on the relative expression across all proteins in the panel. In panels that contain a few very high-expressing antigens, most of the sequence reads can be taken up by a small number of antigens. In this case, the dynamic range of the remaining antibodies is significantly reduced. While there are a number of ways to approach this (including antibody titration and spiking in cold, unlabeled antibody), one approach that will undoubtedly become popular is to first sort populations of cells defined by high expressing antigens using fluorescently labeled antibodies prior to labelling sorted fractions with oligo-antibodies to identify the remaining antigen profiles.

This FACS-assisted sequencing approach ensures an efficient use of sequencing reads and when combined with hashtag antibodies (93) or lipid modified oligo or cholesterol modified oligo (94) (to molecularly barcode each sorted population), it becomes a powerful multi-omics strategy with high-throughput. A comparison of this approach, including its impact on sequencing read allocation, is modeled in Figure 2.

Application of Genomic Cytometry

Since around 2015, there has been an explosion of methods aimed at single-cell genomic characterization. Alongside this, there have been an increasing number of studies making use of scRNA-seq approaches; see review (95). Indeed, following the completion of the human genome project (96), scientists have become increasingly aware that bulk genomic approaches lack the precision to unravel subtle changes at the level of the individual cell. This is critically important in diseases such as cancer and immune disorders where a single rogue cell can be the base of disease. It is also important for the understanding of many developmental processes where single cells give rise to many cells.

(A)

Population selection	Labeling method	Epitope density	Read usage	Detection sensitivity	Cost efficiency
Hashtag-based	Oligo antibodies	High	High	High	$$$$
	Oligo antibodies	Low	Low	Low	

Population selection	Labeling method	Epitope density	Read usage	Detection sensitivity	Cost efficiency
FACS-assisted	Fluorescently labeled antibodies	High	N/A	High	$$
	Oligo antibodies	Low	High	High	

(B)

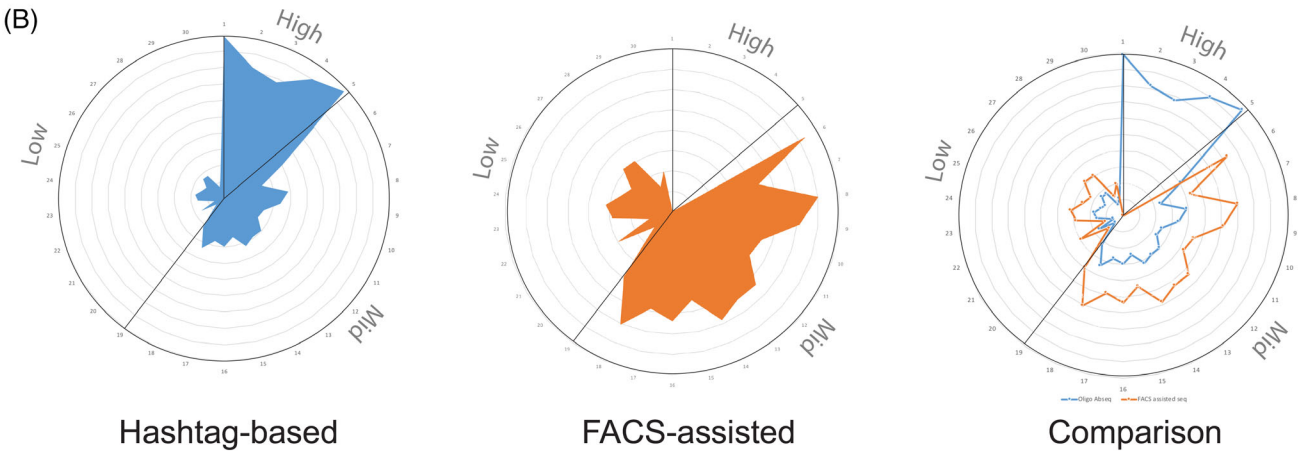

Figure 2. FACS assisted sequencing provides an efficient and targeted multi-omics approach. (**A**) A comparison of standard full oligo antibody panel (unbiased) (top), with FACS assisted sequencing (targeted) (bottom) using a combination of fluorescently labeled antibodies (for pre selection of populations) followed by oligo antibody labelling. (**B**) Read sequencing utilization in a mock panel. An imaginary 30 plex panel was created. The panel consisted of 5 high-expression epitopes, 14 medium-density epitopes, and 11 low expressors. To compare the effect of removing the high-expressing antigens from the sequencing run, we compared the relative proportion of sequencing reads used by each oligo tag under both conditions. Each of the concentric circles in the radar plots indicates a single percentage of sequencing reads used up by the marker. This model predicts that when highly expressed antigens were removed from the oligo antibody panel, it is clear that low-expressing antigens are associated with higher read counts when FACS assisted sequencing was used.

Whole transcriptome analysis, the method used by the majority of scRNA-seq studies to date, allows global profiling of many of the RNA species found in cells in an unbiased manner and without the need of a priori knowledge of the cells or the cell system to be studied. Although single-cell transcriptomic methods are not capable of amplifying every single mRNA, even relatively poorly performing methods are proving capable of accurately identifying many existing and novel cell populations (97). Furthermore, many of the original methodologies are being improved with molecular techniques aimed at increasing transcript detection sensitivity. Notable examples include Chromium V3, Smart-Seq 3, Quartz-Seq2, and Seq-Well S^3 (35, 42, 98, 99).

Early studies have tended to be descriptive efforts, primarily aimed at uncovering the cellular heterogeneity and identifying unique cell types. These studies have formed the basis of the Human Cell Atlas (HCA) project (100). The HCA is a multicenter, international effort aiming to create a database of all cell types in the human body using single-cell approaches. This is an important effort and is the next logical step following on from the human genome project. Just as the human genome project provides the reference data that has allowed deep interrogation of the biology associated with genomic changes, the completion of the HCA should provide the reference data to allow classification of individual cells from their unique omic signatures. This is particularly important, as many of the databases that we are currently using to interpret single-cell genomic studies are based on bulk genomics.

While there is clear virtue in these types of studies, this shotgun approach is only designed to provide a fundamental

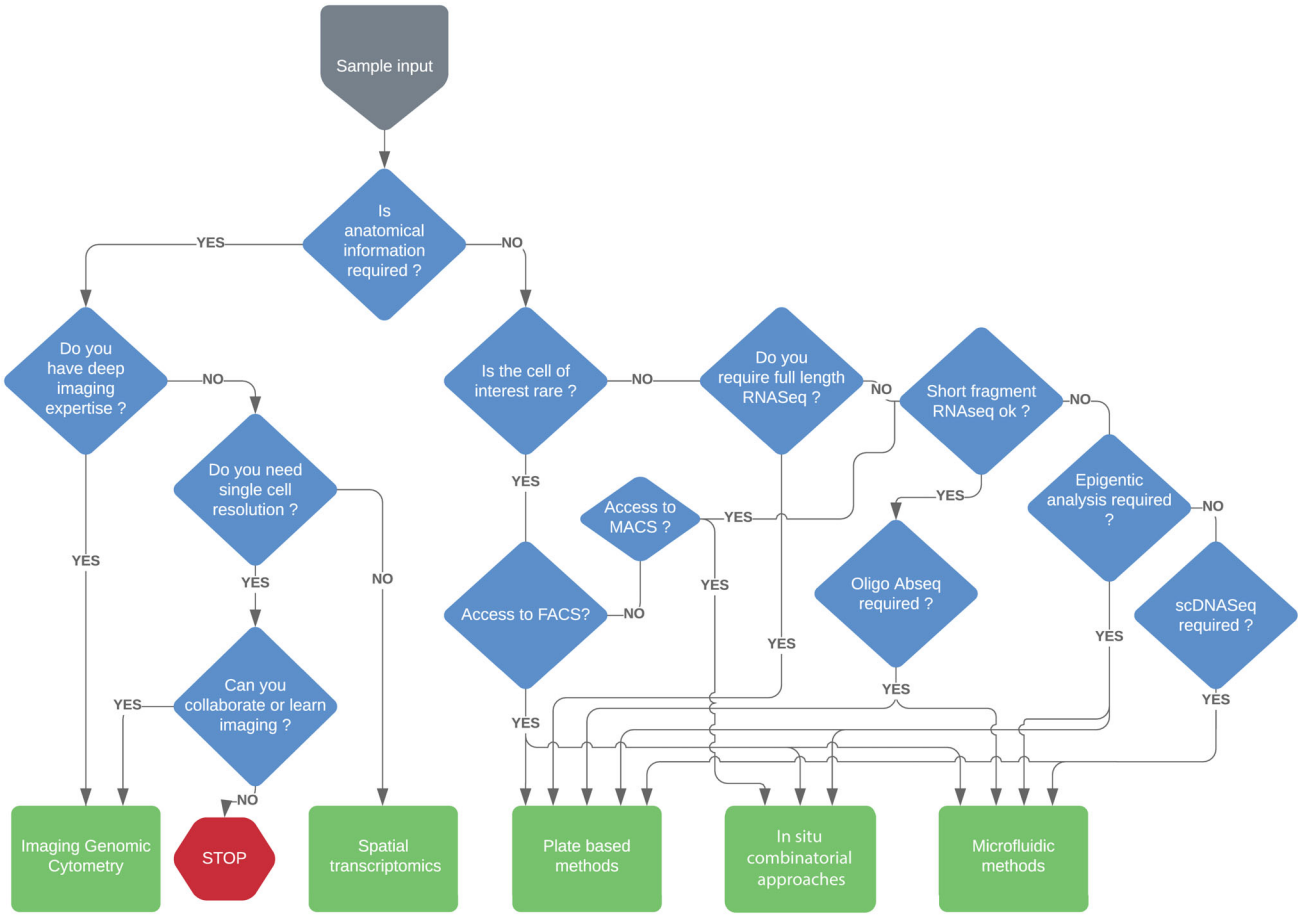

Figure 3. Flowchart for determining the most suitable Genomic Cytometry method for the biological question.

base for more nuanced approaches. The approach required for biologically directed studies will depend on the (1) biology of the system, (2) the questions being asked, (3) the technical expertise of the scientists running the experiment, and (4) the funds available. While the decision of which technology is best suited to the biological question being asked is not always straightforward, we have outlined some of the more common questions involved in the decision-making process in Figure 3.

Conclusion

As we move into the age of Genomic Cytometry, we are now looking to synergistically leverage the modalities of genomics, informatics, microfluidics, and cytometry toward a single aim. To do this, we must develop ways to work in a cross disciplinary fashion such that microfluidics and FACS-based techniques can be seamlessly integrated into molecular workflows and high-dimensional data analysis frameworks. The combination of these four, traditionally distinct expertise areas, is what provides the foundation for the new field of Genomic Cytometry.

With Genomic Cytometry, it is possible to study cellular characteristics more deeply than ever before. The new tools emerging to allow RNA-seq, DNA-seq, epigenetic analysis, and protein detection at the level of the single cell will fundamentally change what we know about biological processes and how quickly we can deeply interrogate complex biological systems. We are beginning to see a systems-based approach that will allow us to do accurate single-cell multi-omics studies with the sensitivity, efficiency, and cost that means true biology can be uncovered. This deep characterization is allowing us to unravel cellular complexity in highly heterogeneous samples and to find the root cause of disease and unravel the cellular complexity of development. Eventually, we believe it will give us the power to analyze the DNA, RNA, protein, and epigenetic states of individual cells at throughputs that will rival that of current flow cytometers.

As the field of single-cell genomics matures, and we begin to embrace the broader field of Genomic Cytometry, it will become increasingly more evident that results from single-cell omics studies will need to be supported and validated by alternate systems. These systems will include traditional imaging, lineage tracing, and fluorescence cytometry methods. This will create a circle of discovery and validation that unites the field of genomics and cytometry. For this reason, although we envision a dramatic shift in the tools

available to the traditional cytometrist, cytometry will still hold a critical place in the emerging application of single-cell genomics. It is, for this reason, Genomic Cytometry will become the modality of choice for single-cell analysis.

Conflict of Interest

The authors declared no potential conflict of interest.

Author Contributions

Luciano Martelotto: Conceptualization; writing-review and editing. **Fatima Valdes-Mora:** Conceptualization; writing-review and editing. **David Gallego-Ortega:** Conceptualization; supervision; writing-original draft; writing-review and editing.

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