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Review Article

From imaging a single cell to implementing precision medicine: an exciting new era

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In the age of high-throughput, single-cell biology, single-cell imaging has evolved not only in terms of technological advancements but also in its translational applications. The synchronous advancements of imaging and computational biology have produced opportunities of merging the two, providing the scientific community with tools towards observing, understanding, and predicting cellular and tissue phenotypes and behaviors. Furthermore, multiplexed single-cell imaging and machine learning algorithms now enable patient stratification and predictive diagnostics of clinical specimens. Here, we provide an overall summary of the advances in single-cell imaging, with a focus on high-throughput microscopy phenomics and multiplexed proteomic spatial imaging platforms. We also review various computational tools that have been developed in recent years for image processing and downstream applications used in biomedical sciences. Finally, we discuss how harnessing systems biology approaches and data integration across disciplines can further strengthen the exciting applications and future implementation of single-cell imaging on precision medicine.

Introduction

Although single-cell and 'omic technologies are relatively young fields that have been developing the past 20 years [1], single-cell imaging in the form of microscopy can be traced back to the 1600s, when Robert Hooke first described the small rectangular compartments he observed with his microscope in cork bark as 'cells' [2]. Little did he know that what reminded him of small rooms in which monks slept in, were in fact the smallest, single units of life, and that his early work was part of the birth of modern microscopy and to an extension, single-cell imaging.

There is no doubt that single-cell 'omic technologies have revolutionized the fields of biology, biomedical sciences and healthcare [3,4]. Scientists can now comprehensively map heterogeneous landscapes of cell types and phenotypes [5], interrogate lineage and state transition dynamics during development and exogenous perturbations (e.g. drug treatments) [6,7] and exploit their power for cell-based diagnostics [8]. Yet, for all their advanced capabilities, they remain tools of indirect assessment that can only infer true behavior, functionality, and changes over time. They also fail to capture and delineate the spatial relationships among cells in their native tissue environment. This is where the power of single-cell imaging remains unmatched [9].

A single cell can be imaged in a variety of ways. The simplest way is with traditional optical microscopy, through which one can visualize the morphology and spatial arrangements of cells in culture or in tissue specimens. Combinations of light and electron microscopy, and advances in super-resolution microscopy techniques in general — not to be discussed in this review — allow imaging of subcellular structures in great detail (see extensive review by Myers et al. [10]). It is however with the advances in live cell fluorescence imaging, microscopy phenomics and multiplexed spatial imaging that scientists have tapped into great potential and high-content cell biology. Following cells in real time, provides dynamic, spatiotemporal insights into how cells grow and change in morphology and behavior under various conditions [11]. High-content, multiplexed spatial imaging technologies allow characterizing

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spatial architecture among cell types and phenotypes within the complex environment of various tissue types and disease backgrounds [12–14]. These latest imaging advancements not only shed light on previously unappreciated facets of cellular biology and function, but also hold promise for therapeutic interventions and diagnostics in healthcare applications.

We describe here the latest advances in high-throughput and spatial single-cell imaging, as well as computational methods that have been developed during the last few years for image processing and analyzing high-content data, with a special focus on the translational applications of single-cell microscopy phenomics and multiplexed proteomic spatial imaging. Finally, we highlight the concepts of systems biology and data integration across other single-cell -omic platforms and discuss how imaging methodologies can further strengthen our efforts on precision medicine as it pertains to diagnostics, prediction models, and therapeutics in healthcare.

High-throughput single-cell microscopy and phenomics: phenotyping cellular behaviors and responses

Advances in hardware automation, quantitative image analysis and machine learning algorithms have helped propel the field from visually studying morphology changes and a small number of cellular parameters (low throughput) to implementing high-content screening of single-cell multi-parametric data [15]. This has led to the emergence of image-based phenomic profiling (Figure 1); an approach where an abundance of heterogeneous cell phenotypic traits can be simultaneously traced and cataloged from multiple samples in response to genetic variations, environmental pressures, and drug treatments [9,15,16]. Phenomics have been empowered by the development of tools such as antibodies, metabolic and oxidative stress sensors [17,18], and fluorescent and bioluminescent reporters [19]. Applications of high-throughput single-cell microscopy (whether 'fixed, endpoint cell assays' or time-lapse imaging) address a broad range of biological queries, from genomic cell cycle studies in fission yeast [20,21] to drug discovery [22], classification, and quantification of the heterogeneity of cancer cell phenotypes under various experimental conditions [23,24]. By incorporating refined technologies such as RNAi, CRISPR, live imaging/optical clonal barcoding, and microfluidics, researchers have the tools to identify new causal relationships between genes and downstream phenotypes and processes like evolution [16,25,26]. They also have the arsenal to track and study dynamic biophysical cellular processes of each cell (e.g. cell shape changes, motility/migration velocity, and stiffness). These live cell phenotypic biomarkers are powerful additions to the traditional protein biomarkers, since through them one could assess and predict

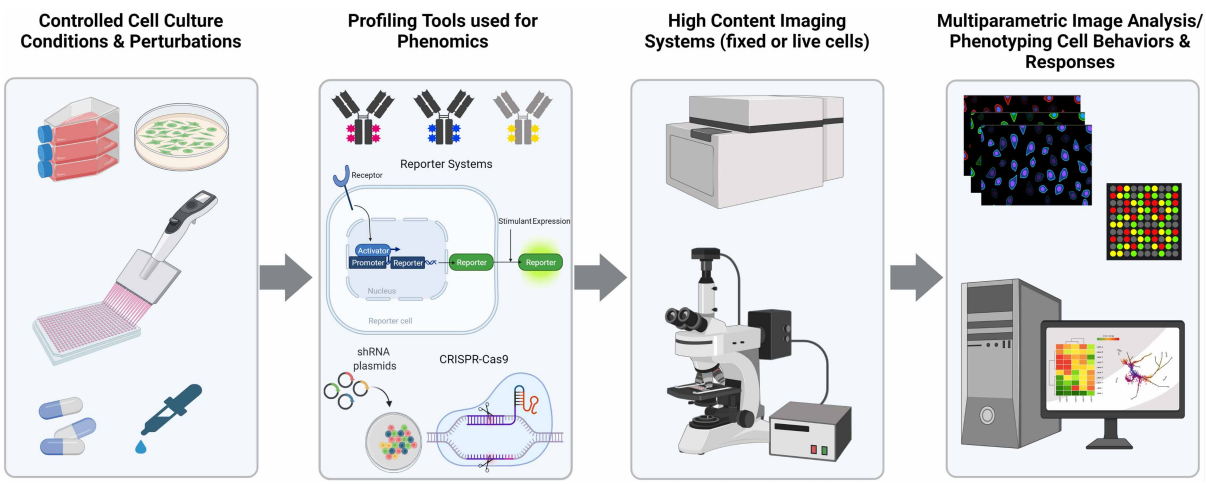

Figure 1. High-throughput single-cell microscopy and phenomics. Schematic overview of image-based phenomic profiling.
By inducing genetic, environmental and treatment perturbations on cells and using high-content screening of single-cell multi-parametric data, researchers can phenotype heterogeneous cell behaviors and responses to various stimuli. Phenomics have been empowered by the development of tools that include antibodies, sensors, reporters, CRISPR and barcoding among others. High-content imaging can be performed on fixed and live cells alike. See text for further details. Created with BioRender.com.

single-cell behaviors and transitions in both normal and disease states and/or under treatment, and therefore could be translated into future clinical use and applied to precision medicine [27].

Single-cell multiplexed spatial imaging: characterizing and mapping cellular phenotypes and architecture in tissue

The majority of high-throughput single-cell microscopy assays are performed on isogenic, isolated cells in multi-well assay plates and not within their native tissue microenvironments, although some do incorporate extracellular matrixes and 3D organoid models to better recapitulate cellular function within a tissue ecosystem [28]. It is now appreciated that the spatial architecture of cell types, cellular signaling phenotypes and tissue structural elements plays a critical role in normal biological processes as well as in disease progression, specifically in cancer [14]. The development of multiplexed spatial imaging platforms allows for characterizing the heterogeneity of cell types and their arrangements within and between tissue specimens in great depth, at both the transcriptomic and proteomic level.

For years, scientists and pathologists have been using histological analysis (hematoxylin and eosin (H&E)) and immunohistochemistry on serial tissue slides to gain insights into biomarker expression, tissue architecture and diagnostics. Along with the rapid growth of single-cell technologies and appreciation of cellular heterogeneity, there has been a growing aspiration to be able to study as many spatially resolved proteomic and transcriptomic markers as possible. Immunofluorescence was the first step that was taken towards multiplex staining, however typically this approach allows for probing a handful of proteins per tissue slide. The explosion of technological advances in a variety of imaging platforms now permits simultaneous interrogation of 30–60 markers and up to 10 000+ targets in spatial proteomic and transcriptomic assays, respectively [14,29]. As of now, spatial transcriptomics do not provide information on localized single cells but rather regions in tissue, as opposed to spatial proteomics, which we will be addressing here.

The expanding field of spatial proteomics is a result of leveraging methods that incorporate iterative cycles of staining and detection, as well as DNA- and metal-conjugated antibodies (Figure 2). In the case of iterative methods, the workflow involves cycles of antibody staining followed by subsequent stripping. Some examples include multiplexed IHC (mIHC) [30] and OPAL staining [31]. Other methodologies, to avoid compromising tissue integrity with many stain/stripping cycles, rather that removing the antibodies themselves, they gently strip an antibody-label instead, like in the case with fluorophore photobleaching (e.g. cyclic immunofluorescence (cycIF [32]). A combination of an iterative staining method and DNA-barcoded antibodies was recently developed, namely the co-detection by indexing (CODEX) which allows detection of ∼50 protein markers [33]. It involves a one-step staining step of the tissue with a pool of DNA-conjugated primary antibodies, followed by iterations of DNA hybridization, imaging and stripping with fluorescently labeled DNA probes complimentary to the probes conjugated to the primary antibodies. CODEX is integrated with a fluorescence microscope that enables slide scanning of whole tissue or tissue microarrays at different resolutions and is also applicable to 3D imaging and staining of thick tissue sections. DNA-barcoded antibodies are also utilized in a similar method called immuno-SABER [34]. Iterative imaging methodologies like CODEX have been able to avoid the limitations of spectral overlap and provide high-plex spatial data, however autofluorescence is a limitation in various types of tissue (e.g lung). Mass spectrometry-related imaging platforms avoid this issue and have the capability of theoretically detecting up to 100 proteins in a single staining and scanning step, avoiding multiple chemical iteration cycles, although the time to scan tissue is significantly longer.

Mass spectrometry-based imaging modalities utilize antibodies conjugated to unique metal isotopes similar to cytometry by time of flight (CyTOF, [7]). Imaging mass cytometry (IMC [35]) and multiplexed ion beam imaging (MIBI [13]) are two variations of mass spectrometry-based imaging [36]. IMC uses a short wavelength beam that is focused on a tissue spot size with a 1 μm diameter. Vaporized tissue of each spot is carried through an inert gas to the mass spectrometer for analysis. MIBI differs in that it uses a focused ion beam and secondary ion mass spectrometry via which it can achieve higher resolution and sensitivity. The tissue is rastered by the primary beam, pixel by pixel. The secondary ions that are then released by the tissue are measured by time-of-flight mass spectrometry (TOF-MS) at defined spatial coordinates, generating an N-dimensional image. Theoretically, the ion beam in MIBI can be reduced to a spot size below 500 nm in diameter to achieve significantly higher resolution. Only a thin layer of tissue can be ablated, thus allowing for multiple scans of varying resolutions in 3D space.

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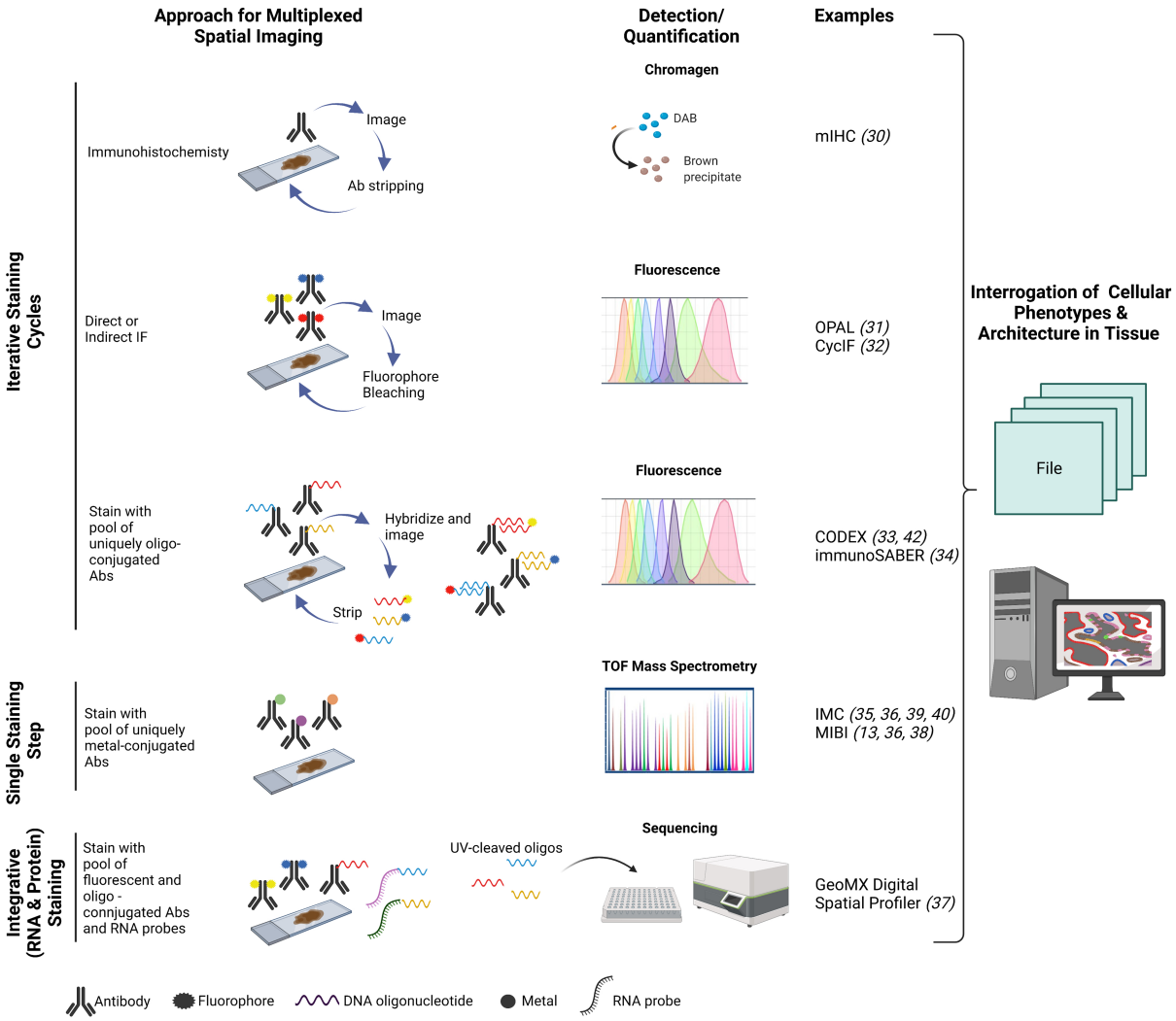

Figure 2. The expanding field of multiplexed spatial proteomics.

Schematic representation and comparison of the most common methods for multiplexed proteomic spatial imaging. Examples discussed in the main text are depicted here, alongside with respective reference numbers in parentheses. mIHC, OPAL, CycIF, CODEX and immunoSABER are some examples of iterative methods that use fluorophore-based detection, except in the case of mIHC (chromagen, DAB). MIBI and IMC use TOF mass cytometry for antibody detection. In the case of GeoMX Digital Spatial Profiler, RNA and proteins can be simultaneously probed with sequencing. Fluorophore-conjugated antibodies used in GeoMX help isolate a region of interest, from which protein and RNA expression will be subsequently analyzed with oligo sequencing. Number of targets that can be interrogated: OPAL (10), ImmunoSABER (10, with capability up to 50), mIHC (30), CycIF, CODEX (60), IMC and MIBI (~40, with capability up to 100). GeoMX can probe ~20–100 targets, with potential of reaching ~800-plex detection of RNA, DNA and protein molecules. In depth description of these platforms and additional examples can be found in the review by Lewis et al. [14]. See text for further details. Created with BioRender.com.

An integrative protein/RNA imaging platform called multiplex digital spatial profiling of protein and RNA (GeoMx Digital Spatial Profiler, Nanostring) was recently developed. Although with limitations when it comes to imaging low-abundance targets in individual cells, it has the capability of detecting ~20–100 proteins, with potential to reach at least 800-plex detection for RNA, DNA and protein [14], enabling researchers to comprehensively interrogate tissue heterogeneity at both the transcriptomic and proteomic level simultaneously [37].

There have been numerous translational studies showcasing the power of single-cell multiplexed imaging in various diseases and in cancer. For example, one study used MIBI to characterize the tumor microenvironment (TME) in triple negative breast cancer (TNBC) from 41 patients and showed an association of architectural

features with survival [38]. IMC has been used to study diabetes [39], profile the immune landscape in multiple sclerosis [40], characterize breast cancer tissue samples [35] and perform multi-dimensional profiling of drug-treated cancer cells [41]. CODEX was utilized to study antitumoral immunity at the invasive front of colorectal cancer [42]. These investigations highlight the importance of interrogating heterogeneous single-cell spatial profiles in the context of their native microenvironment; they also promise to provide a more comprehensive understanding of the complexities of tissue biology and how to harness new knowledge towards research that is more clinically oriented.

Computational frameworks for image processing and analysis of single-cell imaging: from descriptive to predictive biology

Image processing

Perhaps the most challenging part of multiplexed single-cell imaging is handling large images with multimodal data information. High-throughput, time-lapse and spatial imaging data necessitate the establishment of storage capacity and the development of complex software programs and computational tools that are either commercially available/open source or proprietary. A handful of review studies provide comprehensive descriptions on the types and categories of computational analysis that are currently applied in the field depending on the imaging platform [14,36,43,44].

The first step when analyzing imaging data, is performing image processing tasks and these may include separating true signal from noise (background subtraction and denoising), improving resolution, and normalizing for color and intensity variations [44,45]. A variety of methodologies have been applied for image processing across different imaging modalities. For example, noise filtering for MIBI data was performed with a *k*-nearest-neighbor approach in the study by Keren et al. [38]. Deep learning and convolutional neural networks (CNNs) (to be described in more detail below) have been implemented in stain normalization, color standardization and detection of mitosis in H&E slides [46].

Machine learning for segmentation and classification

Machine learning algorithms are often used when a large number of measurements per cell needs to be translated into something that is biologically interpretable. Deep learning and CNNs (which fall under the general umbrella of machine learning approaches), have been heavily utilized and adapted for downstream analysis of multiplexed imaging data, once these pass through the initial processing phase (Figure 3). The concept of these methodologies is inspired by our brain, where the artificial neural network assimilates interconnected neurons grouped into layers. It is comprised by an input layer that aggregates input information/signals from other connected neurons as well as hidden layers using various user-defined weights, and an output layer for predictions of a desired feature [45]. A key drawback of a neural network is that weights are determined by training, which in turn requires massive amounts of annotated data that are often manually established by humans. Furthermore, neural networks consisting of many hidden layers become inherently quite complicated, making interpretation of the data at times challenging. Deep learning and neural networks have been often applied for achieving segmentation of imaging data. Segmentation is the process through which image regions, most often cellular structures and boarders are identified. This step is critical for extracting single-cell data information from an image, especially when imaging tissue and cell co-cultures where various cell types are often overlapping or tightly connected to each other [47]. Examples of segmentation tools include DeepCell, ilastik, Cellpose and Mesmer [48–51]. Segmentation precedes cell annotation and often improves accuracy of downstream classification efforts. Classification deep learning algorithms have been applied in high-content screening microscopy for identifying cell-cycle stages [52], mitotic cells in H&E slides [53] and response to therapeutics [54,55]. They have also been applied to time-lapse imaging for simultaneously detecting and tracking behavior of live cells [56].

Clustering and neighborhood analysis of multiplexed spatial proteomic data

Multiplexed spatial imaging has become an extraordinary tool that illuminates in great detail the complexity of tissue microenvironments in terms of cell positioning patterns, cell-cell interactions and phenotypic heterogeneity. This has led to the expansion of computational platforms like histoCAT, CytoMAP and spatial-LDA [57–59]. These platforms aid in accurately identifying cellular states and phenotypes based on protein expression,

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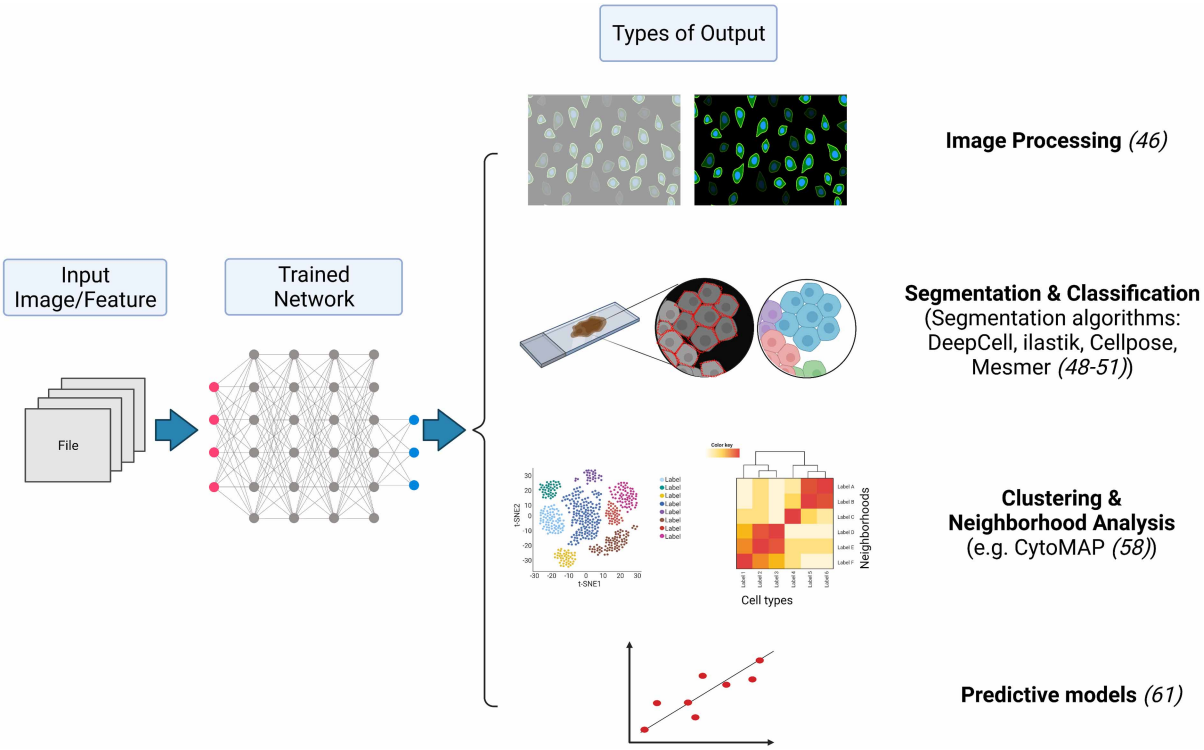

Figure 3. Machine learning approaches for image processing and single-cell imaging analyses.

General schematic showing how deep learning and neural networks are often applied for image processing and single-cell imaging analyses discussed in the main text. Given an input of a high-content image with specific features of interest, a trained neural network can provide an output related to image processing, segmentation/classification of cells, and clustering/neighborhood analysis of cell types found in tissue. Machine learning can also be used for building predictive models (e.g. prediction of drug responses). Numbers in parentheses represent cases in the citing literature in which machine learning was utilized for the specific computational framework. See text for further details. Created with BioRender.com.

mapping cellular neighborhoods and their spatial architecture, and visually presenting multiplex imaging data in a comprehensive way that reveals novel underlying biology.

Predictive models and analysis

The true power of single-cell multiplexed imaging lies in using it for translational research that may impact clinical efforts. The immense, deeply characterized single-cell data being generated by high-throughput microscopy and spatial proteomics can inform predictive models of cellular behavior and clinical outcomes. For instance, Way et al. used simple machine learning algorithms to predict morphology-based health readouts that were generated from the scalable image-based morphology assay, Cell Painting [60] and from publicly available data sets of microscopic images of cells perturbed by thousands of compounds [61]. In another study, high-content imaging parameters were used to predict functional phenotypes in human bone marrow stromal stem cells grown in culture [62]. Deep characterization of the immune microenvironment in longitudinal melanoma tumor samples during treatment using mIHC and gene expression profiling showed that certain immune signatures were predictive of response to immune checkpoint blockade [63]. These studies substantiate the promising translational implications of multiplexed single-cell imaging.

Systems biology approaches and data integration across platforms: the era of precision medicine

In the age of single-cell multi-omic technologies, it has become increasingly evident that systems biology will be fundamental towards gaining a holistic view of any biological *system* we aim to study and understand. As a field, systems biology is inherently multi-disciplinary and therefore best suited to delineate and model *systems-*

wide behaviors of biological ecosystems [64]. It achieves this by implementing computational and mathematical tools designed for analyzing high-dimensional data generated by carefully designed experiments and publicly available datasets. To reach however the lofty goals of puzzling a system's pieces together, integration of the various -omic modalities becomes critical. Omic data can be integrated in three main ways: *horizontal* integration which is used for data sets of the same type; *vertical* integration through which varying data are collected from the same population of cells and *diagonal* integration, where data are collected and integrated across unrelated population of cells [1]. Integrating diverse sets of information between molecular layers (e.g. transcriptomic, epigenomic, proteomic, spatial) can become particularly challenging, as different layers of information may require analytical approaches that are vastly different [65]. Nevertheless, there have been advances in

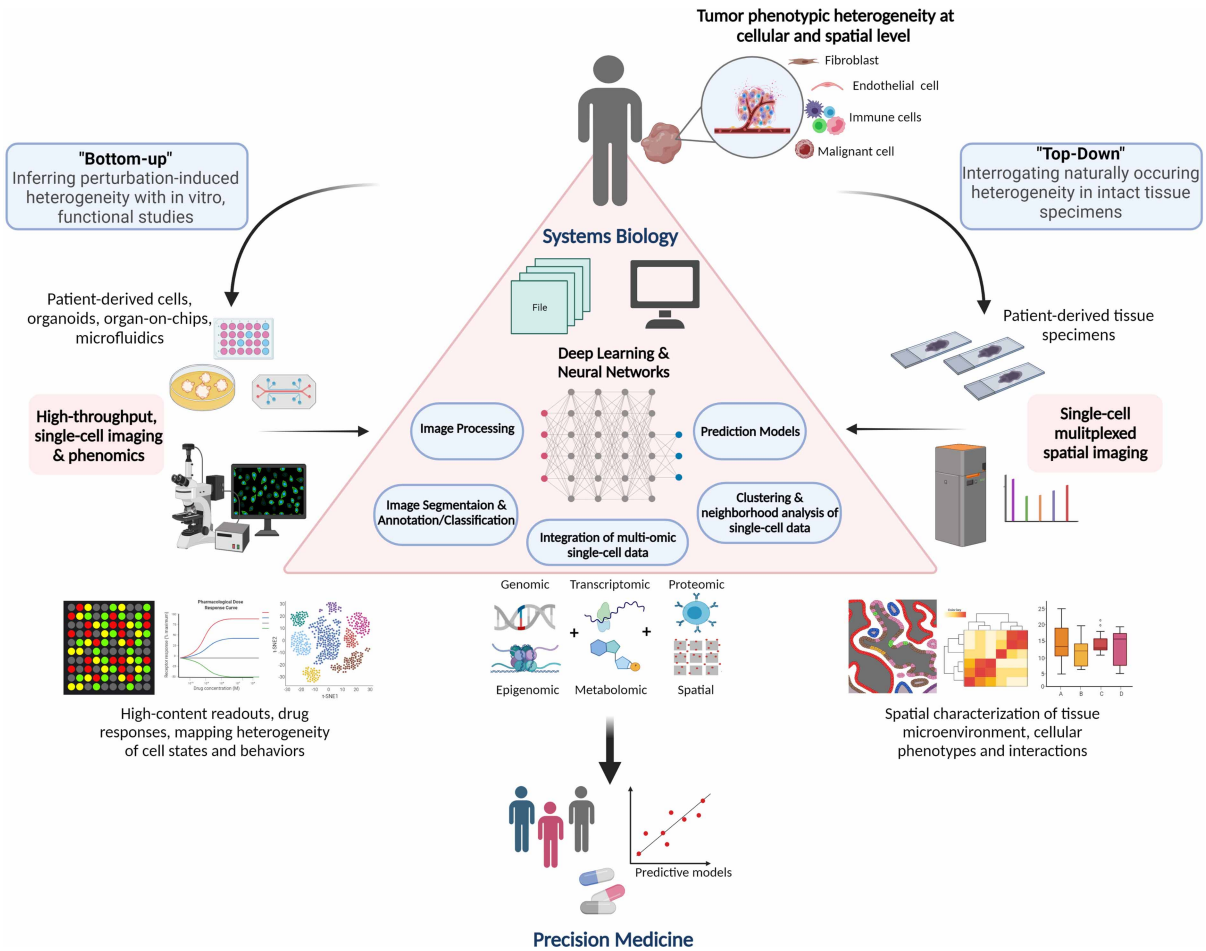

Figure 4. Systems biology approaches, single-cell multiplexed imaging and precision medicine.

Schematic that summarizes topics discussed in this review. It depicts how utilizing systems biology, bottom-up/top-down [71] and specifically single-cell imaging approaches are promising avenues through which the field can begin moving towards the era of precision medicine. Here we illustrate a cancer patient as an example, where patient-derived material can be studied in the lab using bottom-up (high-throughput single-cell imaging and cell-based assays on dissociated cells and/or organoids which allows the characterization of perturbation-induced heterogeneity in *in vitro* studies) and top-down methodologies (single-cell multiplexed spatial imaging — e.g. IMC — on intact tumor tissue, through which we can interrogate naturally occurring heterogeneity in intact tissue specimens). Multi-parametric imaging data generated by these methodologies go through a pipeline of machine learning analyses often shared between single-cell imaging modalities. Highlighted here is also the importance of integrating multi-omic data from diverse platforms in effort to gain a comprehensive, deep understanding of the *system* and its behavior, which in this case is the patient-specific tumor microenvironment. Utilizing these applications on patient-derived samples and leveraging computational tools that focus on mapping tumor heterogeneity and predicting drug response and/or survival outcome, promises to propel efforts towards tailoring treatment at a personalized level and materializing the ultimate aspiration of translational research in precision medicine. See text for further details. Created with BioRender.com.

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integrative computational frameworks designed to harmonize multimodal -omic data. In the case of single-cell imaging, integration of spatial and scRNA-seq data successfully maps diverse cell types in tissue [29]. DBiT-seq offers simultaneous interrogation of transcriptomic, proteomic, and spatial data, and thus, not only does it identify cellular types and their architectural patterns in tissue, but also sheds light on their functional traits in context of their microenvironment, as dictated by protein expression [66]. Very recently, integrating mass spectrometry imaging data (e.g. Matrix Assisted Laser Desorption/Ionization MALDI, time-of-flight secondary ion mass spectrometry/TOF-SIMS) with single-cell IMC or MIBI data has increasingly become an important research focus. This will significantly broaden our understanding on tissue biology and functionality, since we would be able to map onto singular cells biomolecules such as metabolites, drug compounds and lipids [67,68]. This would provide insights into the interplay between tissue architecture, cells and subcellular molecules all woven into the fabric of the histological landscape of interest.

The vast array of technological advances in single-cell imaging present a unique opportunity tailored for precision medicine in various diseases. Whole slide pathology images have been used to identify patients with clinical heart failure by leveraging a deep learning classifier [69]. Machine learning and high content imaging have also been implemented in predicting neurotoxicity in midbrain organoids in a study focusing on Parkinson risk factors [70]. Here, using a cancer patient as an example (Figure 4), one could envision that combining top-down (spatial imaging analysis of patient tissue) and bottom-up approaches (high-throughput screening microscopy on patient-derived cells and/or organoids), we can begin to delineate patient-specific tumor heterogeneity, drug responses and migration potential at the single-cell level [71,72]. With top-down approaches we are able to interrogate naturally occurring heterogeneity in intact tissue, whereas in the case of bottom-up approaches, we can investigate perturbation-induced heterogeneity by utilizing *in vitro*, functional experimentation. By integrating -omic data across scales and modalities and implementing machine learning algorithms, researchers have the toolkits to construct patient-specific spatial maps of fine molecular detail that could serve as reference for response to therapy and disease progression. Furthermore, patient-derived multi-parametric data can be used for stratifying patients, informing prediction models for clinical outcome, and optimizing treatment at a personalized level [73–75].

Conclusions

In many ways, single-cell imaging is the 'original' single-cell technology. It has been used in the form of optical microscopy for centuries, yet we are just beginning to scratch the surface of the immense capabilities in now has to offer, as enabled by the rapid technological growth in microscopy and computational biology. Ironically, we no longer are hampered by the lack of tools to produce single-cell -omic and functional biological data; the real challenge lies in implementing the right complementary approaches to merge the pieces back together and attain a systems view of the biology we aim to decipher. The future seems even brighter and perhaps more complicated, when we begin to envision the potential of integrating additional dimensions to the platforms discussed here. 4D (spatiotemporal) imaging seems to be on the horizon with advancements in monitoring live cellular changes and behaviors in 3D environments [76]. How we would further simultaneously probe such studies with a multitude of markers remains to be seen. What is truly missing from current methodologies, are joint efforts from the scientific community towards utilizing patient-derived cells and explants [77]. The amount and type of data that can be generated from clinical specimens will undoubtedly alter the current course of biomedical sciences and translational research. As single-cell imaging technologies and analytical methods continue to exponentially advance, systems biology approaches will help navigate our way to the pinnacle of precision medicine.

Summary

- Combination of high-throughput microscopy and multiplexed spatial imaging platforms offer in depth views of cellular biology and dynamic behaviors, phenotypic heterogeneity, and tissue architecture at the single-cell level.
- Advances in single-cell imaging and the generation of multi-parametric imaging data have necessitated the development of machine learning and neural network computational frameworks to handle complex features and interpret underlying biology.

- Applications of machine learning tools on single-cell imaging data not only have unmatched descriptive power but can also be employed to predict drug responses and clinical outcome.
- Systems biology approaches and integration of diverse multi-omic data will be key towards unlocking the potential of single-cell imaging platforms on the path towards precision medicine.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

Abbreviations

CNNs, convolutional neural networks; CODEX, co-detection by indexing; IMC, imaging mass cytometry; MIBI, multiplexed ion beam imaging.

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