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In situ characterization of the tumor microenvironment

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The development of new therapies for cancer is underpinned by an increasing need to comprehensively characterize the tumor microenvironment (TME). While traditional approaches have relied on bulk or single-cell approaches, these are limited in their ability to provide cellular context. Deconvolution of the complex TME is fundamental to understanding tumor dynamics and treatment resistance. Spatially resolved characterization of the TME is likely to provide greater insights into the cellular architecture, tumor-immune cell interactions, receptor–ligand interactions, and cell niches. In turn, these aid in dictating the optimal way in which to target each patient's individual cancer. In this review, we discuss a number of cutting-edge *in situ* spatial profiling methods giving us new insights into tumor biology.

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Introduction

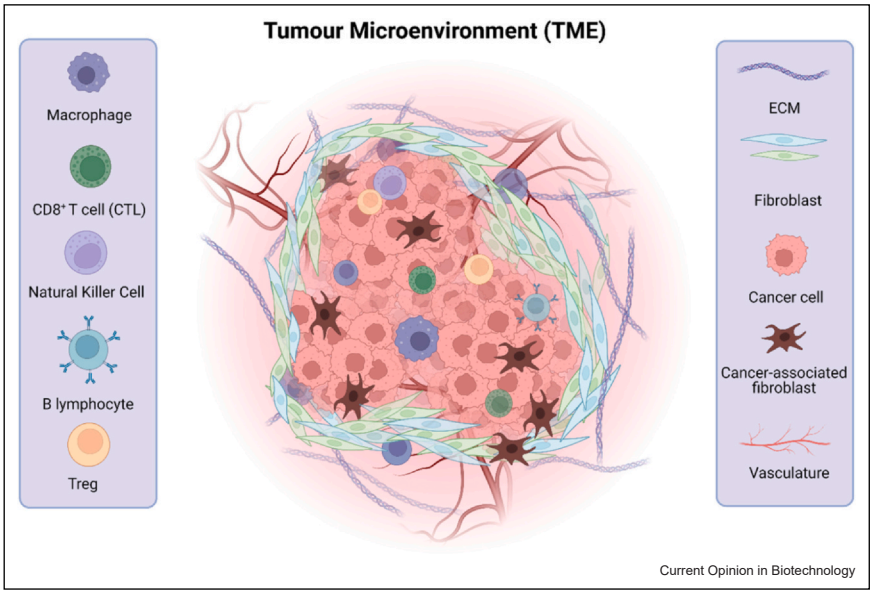
The tumor microenvironment (TME) is a structured milieu of cells in which a tumor exists. The composition of the TME is influenced by immune and nonimmune cells, extracellular matrix (ECM), and soluble and physical features such as acidic pH and hypoxia (Figure 1) [1–3]. The heterogeneity within the TME impedes the development of effective treatment strategies for targeting tumor cells and is thought to be one of the primary causes of treatment resistance and failure [4,5]. The advancement in sequencing technologies has enabled a surge of studies revealing intratumor heterogeneity [6]. It is possible to characterize intratumor heterogeneity at the level of DNA, RNA, and post-transcriptional processes. Many technological and functional advancements in next-generation sequencing (NGS) have led to the implementation of genomics-based approaches in standard clinical practice [7]. NGS has been used effectively to increase our knowledge of cancer genomics, which includes mutational profiling as well as structural aberrations, particularly by whole-genome sequencing and whole-exome sequencing [8,9]. Despite their widespread use, these methods have several drawbacks, including a bulk readout of the TME without cellular resolution or context [9–11]. Single-cell RNA sequencing (scRNA-seq) overcame these challenges and allowed the field to develop transcriptomic data with single-cell resolution, which contributed to the discovery of cell types and states [12,13]. However, scRNA-seq-based technologies are used as input cells that have been dissociated from the original tissue, without providing information about the spatial distribution and location of those cells [14,15]. More recently, spatial profiling technologies enabled the mapping of cells directly *in situ*, allowing for direct labeling and profiling of the TME [16,17]. Nature Methods named 'spatial transcriptomics' the method of the year in 2020 as a 'breakthrough' technology enabling new biological and clinical insights [18]. As a relatively new field with rapid growth and adoption, spatial profiling technologies are still in their infancy. There remain limitations, such as multiplexing, resolution, throughput, and sensitivity [14]. Given the growing number of commercial solutions available today, we anticipate the widespread adoption of these technologies in the coming years in discovery, translational, and clinical studies. Here, we discuss a number of cutting-edge *in situ* spatial profiling technologies and their applications in cancer research (Table 1).

Spatial profiling technologies

Molecular Cartography (Resolve Biosciences)

The Molecular Cartography platform enables the measurement of spatial gene expression patterns at subcellular resolution, without the need for downstream sequencing and enzymatic amplification [19,20]. Using single-molecule fluorescence *in situ* hybridization and transcript-specific probes, the platform generates

Figure 1



The TME composition. The TME surrounds the tumor and consists of various immune and nonimmune cell types, ECM, and physical features such as an acidic pH. Some cell populations, such as CTL and natural killer (NK) cells cells, inhibit tumor growth, whereas others, such as regulatory T cells (Tregs) and cancer-associated fibroblasts, promote tumor progression. Created with BioRender.com.

Table 1

Spatial profiling technologies and applications [19–31,33–37,39–43,45–53,58].

Technology	Methodology	Assay type	Omics	Sample type	Resolution	Throughput	Plexing	Capture area	Advantages	Disadvantages	References
Molecular Cartography	• Single-molecule FISH	• Imaging	• Transcriptomics	• FFPE • Fresh-frozen • Cultured cells	• Single-cell and subcellular resolution in x-,y-,z (300 nm)	• Up to 24 samples per run (3 slides per run, each with an 8-sample capacity)	• 100 RNA transcripts	• 1 cm^2	• Preserving tissue architecture • High throughput	• Limited to 100 RNA transcripts per sample • Requiring specific slides • Limited capture area	[19-25]
PhenoCycler-Fusion	• *In situ* hybridization of fluorescent reporter oligonucleotides to DNA-barcoded tags conjugated to antibodies	• Imaging	• Transcriptomics • Proteomics	• FFPE • Fresh-frozen	• Single-cell and subcellular resolution in x-,y-,z (250 nm)	• 1 slide per run	• 100 RNA transcripts • 100+ protein biomarkers	• 22 × 22 mm	• Whole-slide profiling • Multi-omics profiling • Using standard microscopic slides • End-to-end solution • Large capture area	• Limited to 100 RNA transcripts per sample • Targeted profiling	[26-31,33-35]
Xenium	• *In situ* sequencing (ISS) using DNA padlock probes	• Imaging	• Transcriptomics • Proteomics	• FFPE • Fresh-frozen	• Single-molecule detection with subcellular resolution in x-,y-,z (200 nm)	• Up to 6 slides per week (2 slides per run)	• 1000 RNA transcripts • Multiplexed protein biomarkers	• 12 × 24 mm	• No need for downstream sequencing for RNA profiling • End-to-end solution • Onboard analysis happens in parallel to instrument run • Multi-omics profiling	• Low throughput • Targeted profiling • Requiring specific slides	[36,37,39,58]
CosMx SMI	• Cyclic *in situ* hybridization (ISH) of barcoded fluorescent reporter probes	• Imaging	• Transcriptomics • Proteomics	• FFPE • Fresh-frozen • Cultured cells • Organoids	• Single-cell and subcellular resolution in x-,y-,z (100 nm)	• Up to 16 slides per week (4 slides per run)	• 6000 RNA transcripts • 124 protein biomarkers	• Desired imaging area is 100 mm^2 (up to 375 mm^2)	• 3D mapping with subcellular resolution • End-to-end solution • Cloud-based computing and storage • Using standard microscopic slides • Large capture area • Multi-omics profiling	• Targeted profiling	[40-43]
MERSCOPE	• Multiplexed error-robust fluorescence *in situ* hybridization (MERFISH)	• Imaging	• Transcriptomics • Proteomics	• FFPE • Fresh-frozen • Cultured cells	• Single-cell and subcellular resolution (100 nm)	• 1 sample per run	• 500 RNA transcripts • 6 protein biomarkers	• 1 cm^2	• No need for downstream sequencing for RNA profiling • End-to-end solution • Interactive software for visualizing MERFISH data, including cells and detected transcripts	• Targeted profiling • Requiring specific slides	[45-50]
Curio Seeker	• DNA-barcoded beads	• NGS	• Transcriptomics	• Fresh-frozen	• Single cell (10 μm)	• Up to 20 samples per run	• Whole transcriptome	• 3 × 3 mm	• Whole transcriptome profiling • No tissue optimization • No specific hardware	• Only applicable for transcriptomics • Not applicable for FFPE tissues • Limited profiling areas • Limited subcellular resolution	[51-53]

expression data for up to 100 genes and is able to detect rare transcripts [21]. Following tissue sectioning, the transcript-specific probes are hybridized, and iterative cycles of probe colorization, imaging, and decolorization are performed to detect all transcripts. The technology uses its own microscope slides with a capacity for eight samples, and since the platform can run three slides at once, 24 samples can be processed at the same time. The capture area covered by the instrument is 1 cm^2. The instrument can map transcripts in three dimensions with subcellular resolution (300 nm) [22,23]. Molecular Cartography can detect transcripts with a length of at least 700 nucleotides, which is the length of short mRNA molecules such as cytokines [21]. Ghasemi et al. employed both single-nucleus RNA sequencing and spatial transcriptomics to characterize the genetic basis of medulloblastoma with extensive nodularity (MBEN) [24]. They were able to cluster MBEN cell stages, including proliferating and nonproliferating early cerebellar granular neuronal precursor (CGNP)-like cells, migrating

CGNP-like cells, and neuronally differentiated tumor cells. Furthermore, a spatial analysis of the above clusters revealed that early CGNP-like cells formed the internodular compartment, while neuronally differentiated tumor cells formed the nodular compartment [24]. Also, using the platform, Karras et al. mapped the distribution of melanoma cell states and their interactions with TME cellular components. They revealed that tumorigenic competence supporting primary tumor growth could be acquired by melanoma cells following exposure to specific signals from endothelial cells in spatially localized perivascular niches [25••]. They also characterized the phenotypic heterogeneity of melanoma cells, showing that a distinct population of cells contributed to metastatic spread rather than primary tumor growth.

PhenoCycler-Fusion (Akoya Biosciences)

The PhenoCycler-Fusion (formerly CO-Detection by indEXing [CODEX]) is a platform that can visualize multiplexed proteins and RNA molecules in a tissue sample. In this method, antibodies conjugated to DNA-barcoded tags are used to label proteins in the tissue [26]. During imaging, fluorescent reporter oligonucleotides are hybridized to the tags attached to the antibodies. Three fluorescent reporters are detected during each scan, and through iterative cycles of scanning, imaging, and removing, this process continues until all biomarkers of interest are imaged (Figure 2a) [27–29]. The PhenoCycler-Fusion system is paired with the RNAscope HiPlex v2 assay, which uses RNAscope *in situ* hybridization (ISH) technology to detect RNA transcripts, in order to enable the combined analysis of both protein and RNA [26,28]. The instrument can be applied to formalin-fixed paraffin-embedded (FFPE) tissue, fresh-frozen tissue, and tissue microarrays. This platform enables the imaging of every cell across an entire tissue, allowing for *in situ* cell phenotyping at single-cell resolution [30,31••]. With the ability to detect more than 100-plex RNA and protein biomarkers [32••,33], the PhenoCycler-Fusion possesses a multi-omics capability. One of the most significant advantages of the instrument is whole-slide profiling, which eliminates the need to manually select regions of interest, thereby avoiding biased selection and profiling of regions within tissues [34]. By presenting single-cell and subcellular resolution down to 0.20 µm [33], the PhenoCycler-Fusion leads to the discovery of rare cell types as well as the dissection of the spatial neighborhood, signatures, and activation states in the TME. Shekarian et al. used CODEX to investigate the response to 7 days of *ex vivo* immunotherapy with anti-CD47 and/or anti-PD-1 in glioblastoma (GBM) explants from the tumor center and tumor periphery [35]. After treatment of the samples with the immunotherapy agents, they were able to identify the spatial location and distribution of over 850 000 cells. The researchers found that immunotherapy-treated tumor center explants had an enrichment of $CD4^+$ and $CD8^+$ T cells (CTL) relative to the untreated control, and concluded that *ex vivo* immunotherapy of GBM explants could activate antitumor immune response within the tumor center [35].

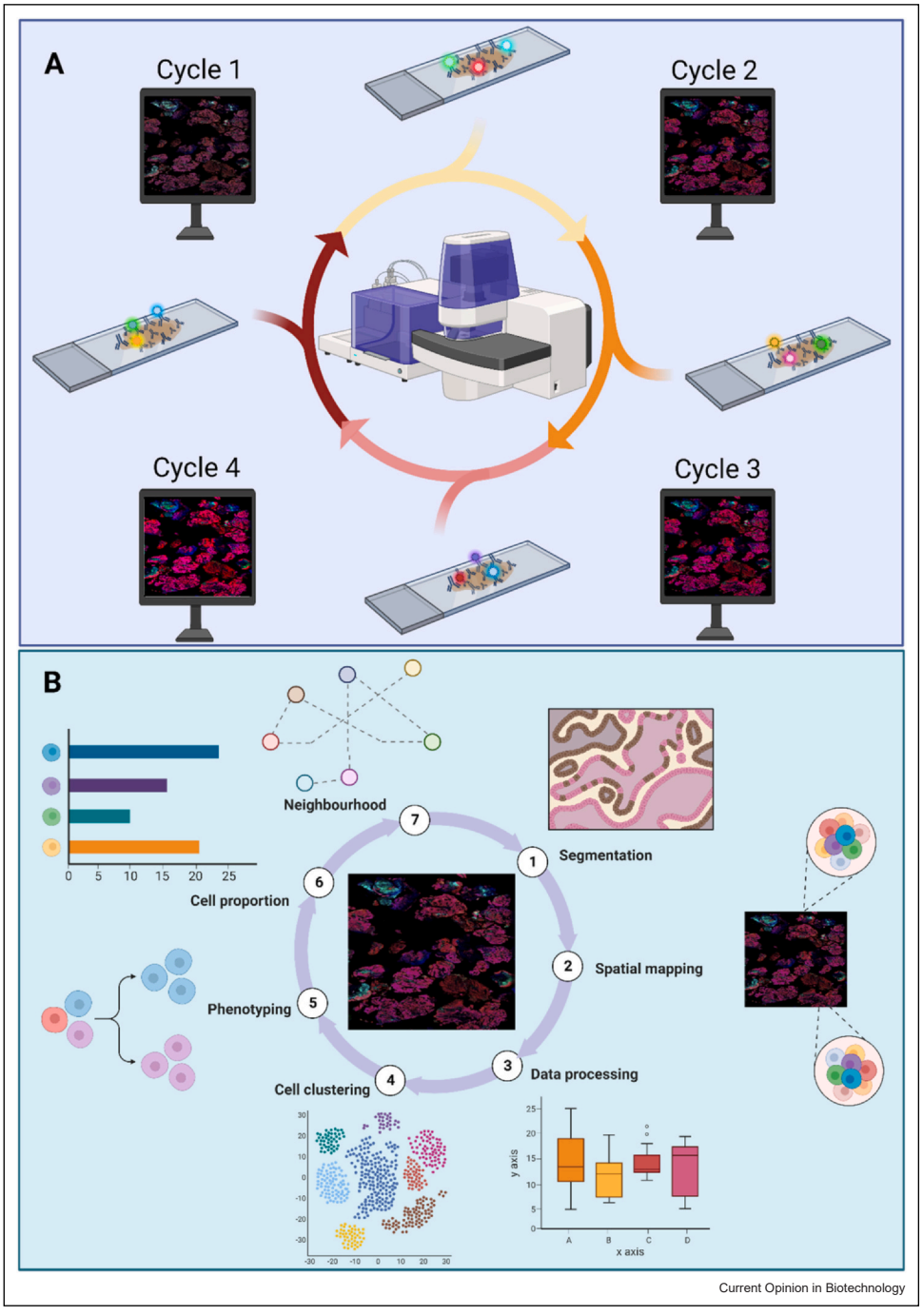
Xenium (10x Genomics)

A new platform developed by 10x Genomics, called Xenium *In situ*, enables single-cell and spatially resolved gene and protein expression analysis in tissue samples [36]. The Xenium platform has the ability to map thousands of RNA transcripts with cellular and subcellular resolution (200 nm) with Z-dimension information [37]. Recent announcements at the Advances in Genome Biology and Technology (AGBT) 2023 conference have indicated a roadmap to achieve 5,000-plex RNA analysis [26]. The platform performs *in situ* sequencing (ISS) using successive rounds of probe-based hybridization, imaging, and removal, to present the precise cellular localization of RNA molecules within each tissue [14,38]. Padlock probing, rolling circle amplification (RCA), and sequencing-by-ligation are the three techniques that are utilized in the process of ISS [36]. The padlock probe has sequences at its 5'- and 3'-ends that are designed to hybridize to the target mRNA. RCA replicates the padlock probes whose 5'- and 3'-ends have both hybridized to the target gene and undergone successful ligation, resulting in the generation of concatenated copies of the probes [14,38]. This method allows for the identification of cell types and their locations within their biological context, as well as information about cellular neighborhoods and communications [37]. From a technical standpoint, it is noteworthy to mention that Xenium covers an imaging area of 12×24 mm. Furthermore, the platform has the capability to execute three runs per week and process two slides during each run. Janesick et al. performed high-resolution mapping of the TME in FFPE tissues from breast cancer patients [39]. For this, they used a 313-gene panel, and they were able to visualize 167 885 cells as well as around 37 million transcripts, with a median of 166 transcripts per cell [39].

CosMx Spatial Molecular Imager (NanoString Technologies)

The CosMx Spatial Molecular Imager (SMI) technology from NanoString offers spatially resolved RNA and protein profiling, with single-cell and subcellular resolution (100 nm) [40••]. The CosMx SMI is compatible with both FFPE and fresh-frozen tissues [40••,41]. The technology's key feature is high-plex multi-omics profiling, which has been demonstrated to be class-leading at 980-plex RNA and 108-plex protein molecules in tissue samples [40••]. Announcements at the AGBT 2023 conference showed 6000-plex RNA and 124-plex protein measurements in early studies [26]. The SMI technology utilizes ISH probes to bind to target RNA molecules and to oligonucleotide-conjugated antibodies,

Figure 2



The workflow of spatial profiling approaches. **(a)** *Akoya Biosciences' PhenoCycler-Fusion proteomics workflow*. Tissue slides are prepared and stained with antibodies conjugated to DNA-barcoded tags. To image the antibodies, fluorescent reporter oligonucleotides are hybridized to the tags. Then, the iterative cycles of hybridizing–imaging–removing are performed. Three fluorescent reporter oligonucleotides are imaged in each cycle, and this process is repeated until all biomarkers of interest are imaged. **(b)** *Computational spatial profiling analysis*. To obtain single-cell spatial information, several steps must be completed after data generation, including (1) cell segmentation, (2) spatial mapping, (3) data processing (quality control, normalization, and batch correction), (4) cell clustering, (5) cell phenotyping, (6) calculation of cell proportion, and (7) definition of cellular neighborhoods. Created with BioRender.com.

which detect target proteins. Sets of barcoded fluorescent reporter probes that bind the ISH probes are then cyclically applied, imaged, and removed to enable the visualization of RNA and protein molecules for the localization and profiling of gene and protein expression [40–43]. One of the platform's distinctive characteristics is the capacity to capture a large scan area on tissue slides (up to 375 mm^2), enabling imaging of large tumor tissue sections [40–42]. The SMI instrument can process four samples per run and sixteen samples per week. The instrument is also capable of mRNA gene expression mapping in three dimensions [43]. He and colleagues investigated RNA and protein expression in the TME of non-small-cell lung cancer and breast cancer tumors [40••]. They were able to profile 980 RNA targets and 108 protein targets within the tissues, and discovered more than 18 distinct cell types as well as 10 unique TMEs in the tumor samples, with single-cell and subcellular resolution [40••].

MERSCOPE (Vizgen)

MERSCOPE is an instrument based on multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) [44] technology that employs error-robust barcoding chemistry to directly image RNA transcripts without the need for downstream sequencing [45,46]. MERSCOPE produces high-resolution spatially resolved images of RNA molecule distributions with single-cell to subcellular resolution (approximately 100 nm) [47,48]. Using a custom gene panel, the current chemistry of the platform can simultaneously map up to 500 genes [46,49] and also 6 protein biomarkers [50]. To detect rare transcripts, MERSCOPE employs multiple fluorescent probes for a single transcript to enhance detection sensitivity [47]. The technology employs interactive software to visualize MERFISH data, which includes cells and detected transcripts [45]. For imaging, it should be mentioned that MERSCOPE exclusively employs its unique proprietary slides. These slides necessitate access to the tissue blocks and possess a capture area of up to 1 cm^2 [46]. Using the MERSCOPE technology, Emanual and colleagues revealed the transcriptional organization of the mouse brain and found the positions of 554 802 908 RNA transcripts from 483 genes within 734 696 cells [47••]. As a result, a map called the MERFISH Mouse Brain Receptor Map was created [47••], allowing researchers to investigate the cellular, subcellular, and functional organization of an intact brain.

Curio Seeker (Curio Bioscience)

Curio Seeker is the commercial product of the Slide-seqV2 technology, which uses arrays of DNA-barcoded beads to generate transcriptome-wide maps of RNA molecules in tissue samples [51]. Tens of thousands of beads, each carrying a unique DNA barcode denoting the position of that bead, are arrayed on a slide. RNA from an overlaid tissue section is captured on the array of spatially barcoded beads. By linking the unique spatial DNA barcode of a bead to the transcripts captured by that bead, Curio Seeker allows the position of each transcript to be inferred by sequencing. Curio Seeker provides a near-cellular spatial resolution of 10 µm, which is the diameter of each barcoded bead on the array [51,52]. The technology is distinguished in that no specialized hardware is required; all the user needs is a Curio Seeker slide containing a 3 × 3-mm tile with the spatially barcoded beads [51,53,54]. Spatial tissue analysis is performed by mounting a tissue section on the slide, capturing the RNA from the tissue on the spatially barcoded beads by hybridization, performing reverse transcription for first-strand cDNA synthesis, dissociating and recovering the beads from the substrate, amplifying the cDNA for NGS library preparation and sequencing, and then mapping the barcoded reads to their position of origin to create a transcriptome-wide spatial map of expression. With the standard single-cell sequencing workflow, up to 20 samples can be processed at the same time. The Curio Seeker workflow requires roughly 8 h, with 2.5 h of hands-on time [53,55]. Using Slide-seqV2, Hirz et al. investigated the TME characteristics of prostate cancer. The study found that the immunosuppressive TME of prostate cancer included exhausted T cells, suppressive myeloid cell populations, and a high level of stromal angiogenic activity. When Slide-seqV2 and scRNA-seq were compared, a substantially higher fraction of epithelial cells and fibroblasts and lower fraction of immune cells were measured in prostate tissue samples analyzed with Slide-seqV2 versus scRNA-seq [53].

Conclusions

Within a short period of time, the spatial biology field has evolved from a multicellular- to a single-cell resolution readout. This exponential growth in technologies has come about at an astounding pace, facilitated by the rapid development of *in situ* technologies. To analyze the data being generated by *in situ* spatial profiling tools, computational approaches are being developed to address the complexities around imaging-based readouts, transcript localization, integration of data across multiple planes (x/y/z), cell segmentation, cell classification, and data cleaning (Figure 2b). Numerous factors, such as the analyte(s) (RNA, protein), readout (imaging, sequencing), throughput (samples per day/week), number of targets to be counted (plexing), and resolution (cellular or subcellular), need to be considered to determine the optimal *in situ* spatial profiling technology to apply to each use case. The spatial profiling toolkit is being further expanded by spatial epigenome and other 'omic' mapping modalities coming to the fore, such as spatial T- and B-cell receptor sequencing [56]. Taken together, these cutting-edge biomedical tools could lead

to new discoveries with the potential for translational/clinical assay development. Recent examples of companion diagnostic assays and 'spatial scores' illustrate how these technologies span the breadth of life science and clinical applications [57]. With momentum on both discovery sciences and translational studies, *in situ* spatial technologies will help to drive the next revolution in the life sciences.

CRediT authorship contribution statement

Habib Sadeghi Rad: Writing – original draft; Writing – review & editing. **Vahid Yaghoubi Naei**: Visualization; Writing – original draft. **Majid E. Warkiani**: Conceptualization; Visualization; Writing – review & editing. **Ken O'Byrne**: Conceptualization; Resources; Writing – original draft; Writing – review & editing. **Arutha Kulasinghe**: Conceptualization; Resources; Supervision; Writing – original draft; Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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