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Review

Spatial mapping of cancer tissues by OMICS technologies

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ABSTRACT

Spatial mapping of heterogeneity in gene expression in cancer tissues can improve our understanding of cancers and help in the rapid detection of cancers with high accuracy and reliability. Significant advancements have been made in recent years in OMICS technologies, which possess the strong potential to be applied in the spatial mapping of biopsy tissue samples and their molecular profiling to a single-cell level. The clinical application of OMICS technologies in spatial profiling of cancer tissues is also advancing. The current review presents recent advancements and prospects of applying OMICS technologies to the spatial mapping of various analytes in cancer tissues. We benchmark the current state of the art in the field to advance existing OMICS technologies for high throughput spatial profiling. The factors taken into consideration include spatial resolution, types of biomolecules, number of different biomolecules that can be detected from the same assay, labeled *versus* label-free approaches, and approximate time required for each assay. Further advancements are still needed for the widespread application of OMICs technologies in performing fast and high throughput spatial mapping of cancer tissues as well as their effective use in research and clinical applications.

1. Introduction

Spatial mapping of nucleic acids, proteins and metabolites in tumor tissues can help in understanding the molecular mechanisms involved in tumorigenesis, early detection of cancers and initiating possible preventative measures. The conventional methods of molecular profiling and tissue imaging of tumors can measure only a few targets at a time and hence are low throughput [1–3] while other technical challenges include difficulties in the collection of samples manually at multiple sites of the tissue [4,5]. In addition, profiling of the tissue through molecular approaches is usually based on the bulk transcriptome analysis methods, which can perform only average gene expression profiling of all sets of cells populations and can provide only limited information about the diversity of cell types in tissue samples. Furthermore, bulk transcriptome analysis methods require dissociation of tissue samples that result in the loss of tissue morphology and spatial information [6]. Although significant progress in imaging-based approaches is made in obtaining spatial information, molecular omics-based profiling is still limited, as few biomarkers over a tissue slide can be obtained due to the limited number of fluorescent channels. Omics technologies such as genomics, transcriptomics, proteomics, metabolomics, and glycomics, primarily aimed at detecting genes, mRNAs, proteins, metabolites and glycomes, are tools that can analyze the structure and functions of the whole makeup of a given biological tissue. The analysis of molecules within tissues is performed with various types of OMICS techniques including DNA microarray analysis, next-generation sequencing technologies, imaging mass spectrometry in proteomics, nanotechniques in proteomics, and high-resolution NMR-based metabolomics, as well as mass-spectrometry based non-targeted metabolomics and metabolome analysis by capillary electrophoresis-mass spectrometry. They can enable the analysis of cell attributes in their natural settings allowing us to increase our knowledge at the single-cell level in a complex landscape

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of tissue microenvironments. Thus, the spatial mapping of tissue analytes with high-throughput OMICS techniques is a promising method for understanding the cellular and molecular heterogeneity in tissues [7]. Over the last few decades, OMICS technologies have progressed to offer highly robust and sensitive methods for molecular analysis of tissue histology, intricate intracellular processes, and intercellular communications [8,9]. Today, digital pathological techniques have been developed that integrate high-resolution imaging methods and prevailing multi-scale technologies to achieve more objective, faster, and accurate analysis as compared to conventional microscopy techniques [10,11]. OMICS methods which were commonly used for average profiling of gene expressions in tissue samples have now found their potential to perform spatial profiling of tissue samples. However, there are inherent hindrances in the application of these approaches for the spatial analysis of biomolecules which will be resolved with the advancement of OMICS platforms. Modern OMICS technologies offer various types of advantages for the analysis of various types of analytes with improved resolutions. For instance, single-cell RNAseq can carry out >10,000 single-cell gene expression analyses for the characterization and the discovery of specific types of cells in tumor tissues [12]. Spatial transcriptomics is used in the spatial profiling of whole transcriptome and sequence information of transcriptome of tissue samples [13]. It is suitable for the analysis of tumor microenvironments and tumor heterogeneity; however, it is time-consuming and still at an early phase of development. Fourth-generation RNAseq has a great potential for *in situ* sequencing as well as displaying prospects in spatial mapping, however, further efforts are needed to properly develop it for spatial mapping of intact tissue samples [14]. Another OMIC technology available is digital spatial profiling (DSP) that is useful for formalin-fixed, paraffin-embedded (FFPE) tissue samples and can perform spatial profiling of cellular components across a biopsy sample; nonetheless, DSP cannot reveal sequence information and is restricted by the small number of gene in a panel [15]. The detection of RNAs and proteins in the case of DSP depends upon hybridization-based approaches and requires probes such as DNA and antibodies. Meanwhile, fluorescent *in situ* hybridization (FISH) can detect only RNAs as the detection of proteins usually does not need the binding of multiple probes and mass spectrometry methods are more developed for mapping of proteins only.

Despite the recent advancements in various OMICS technologies, there is a huge demand for the development and improvement of existing high-resolution multi-omics approaches that are associated with multidimensional measurements [16,17]. OMICS technologies developed over the last few years can simultaneously quantify and map entities like mRNA expressions and protein abundances across the tissue samples in a spatial manner. Among OMICS technologies, molecular profiling displays enough capacity for high-throughput profiling of entities while imaging technologies could explore individual cells and corresponding morphological features in positional context. Together, these approaches possess unlimited potential for the thorough characterization of cellular entities and their spatial organizations.

This review presents a comprehensive explanation of recent advancements in OMICS techniques used for spatial mapping of biomolecules in cancer tissues. A detailed literature survey has been carried out on spatial resolution, numbers of different molecules detected from the same assay, label *versus* label-free approaches, types of molecules detected, and time needed for each assay. It is expected that this review article will help researchers to consider different challenges faced by the existing OMICS technologies to devise new strategies for high throughput spatial profiling of cancer tissues. A schematic displaying basic steps used for spatial mapping of tissues is shown in Fig. 1.

2. OMICS technologies used for spatial mapping of cancer tissues

OMICS technologies aimed at the spatial analysis of biomolecules

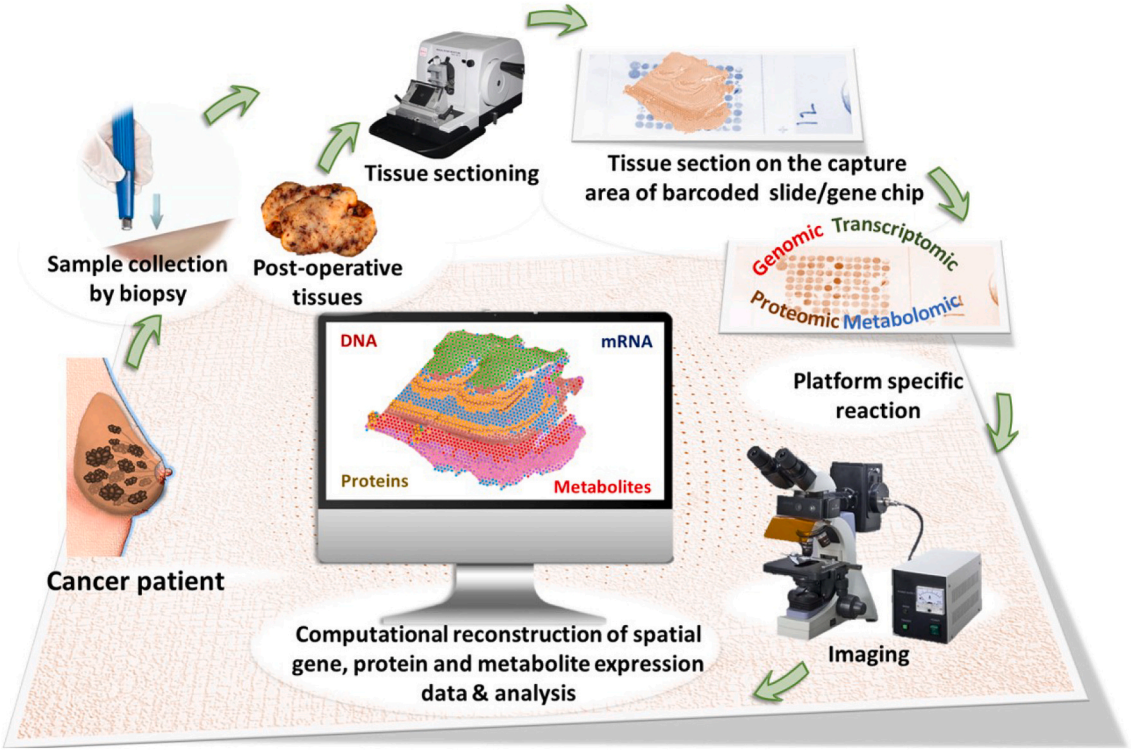

Fig. 1. Schematic showing different steps in the spatial analysis of genomic, transcriptomic, proteomic or metabolomic information from a patient. It involves a series of steps such as the collection of a tissue sample by a biopsy from the region of interest, sectioning of tissue sample into micron-sized thin slices, loading of tissue slices on barcoded slide/microchip, specific reaction or tagging of analytes with a fluorescent probe, capturing of data of interest by imaging, computational processing of data and the reconstruction of spatial information by digital data analysis.

have seen considerable progress in terms of their resolution and robustness in recent years. For instance, localization of DNA in spatial context began with advances in microscopic imaging particularly through super-resolution microscopy as summarized by Xu and Liu [18]. Mass spectrometry has been a commonly used method for profiling the cellular entities especially the proteins in their positional organization in different types of tissue samples [19]. Some of the important studies targeted for profiling of tissue samples included those of Grzelak et al. where the team used Fourier transform infrared (FTIR) spectroscopy for studying the spectral mapping of lipids, proteins, and nucleic acids in ovarian cancers and found that diversity of these analytes play important role in the development of tissue heterogeneity [20]. Su et al. used photocatalytic technology to derivatize unsaturated lipids and then to determine the position of the C=C double bond while Huang et al. worked on the distribution of metabolites in the development of tumor heterogeneity [21,22]. Nilsson et al. applied imaging mass spectrometry for determining the spatial distribution of drugs in cancer tissues which is considered an important hallmark for understanding the pharmacokinetic and pharmacodynamic properties of different drugs [23]. Stahl et al. introduced the term Spatial Transcriptomics where they combined gene expression data with the histological information from the tissue section [24]. Giesen et al. performed spatial localization of 32 proteins by the application of mass cytometry at the subcellular level and showed that these proteins biomarkers are mainly involved in tissue heterogeneity in cancer tissues [25]. Spatial localization of entities through digital spatial profiling approaches was initiated in recent years with expectation of performing pixel-by-pixel analysis of tissue imaging slides [11]. Moreover, the integration of digital pathological techniques with machine learning algorithms had established its uniqueness in mapping multidimensional spatial localization of biomolecules for a broader understanding of intercellular or cell-matrix interactions and tissue heterogeneity [26]. A historic timeline of spatial mapping of tissue samples performed with OMICS technologies is shown in Fig. 2.

3. Approaches and techniques used in OMICS technologies for spatial mapping

Spatial profiling of entities such as mRNA or proteins in intact tissue sections is performed either through image-based technologies or sequencing-based technologies. Both technologies are based on different principles and strategies which confer them different features for spatial profiling of analytes. Some of the examples of imaging-based technologies include single-molecule fluorescence *in situ* hybridization (smFISH) [1,2], sequential barcoding technique [27], seqFISH+ [28], multiplexed error-robust FISH (MERFISH) [29], osmFISH [30], spatially resolved transcript amplicon readout mapping (STARmap) [31], GeoMx Digital Spatial Profiler (DSP) [32], imaging mass cytometry [25,33].

Spatial profiling of transcriptome activities has been achieved by the application of sequencing-based technologies. For a long time, analysis of RNA expression in their original position remained a major challenge even though the detection of RNA through a transcriptome-wide approach had been well established. The addition of barcodes into the target sequences before the extraction of RNA from tissue helped to overcome this limitation. This approach had led to the development of a number of strategies such as spatial transcriptomics (ST) technology [6] and was later acquired by 10× Genomics. Slide-seq [34], Drop-seq technology [35], and high definition spatial transcriptomics (HDST) [36] are another set of OMICS technologies used for localization of RNA analytes in tissue samples with improved sensitivities and enhanced resolutions. There has been huge progress in the field of Slide-seqV2 [37], DBiT-seq [38], Seq-scope [39], STRP-seq [7], Pixel-seq [40], stereo-seq [41] for the spatial localization of RNA and proteins even to single-cell level resolutions over the last few years.

An overview of some of the recent OMICS techniques used for molecular analysis of cellular entities is presented in Table 1.

4. OMICS technologies used for the spatial mapping of cancer tissues

Different types of OMICS technologies such as microarray technology, mass spectrometry, fluorescent *in situ* hybridization and digital pathology-based technologies have been utilized for the analysis of cancer tissues. Here we will focus on OMICS technologies that were initially used for profiling the molecular components of tissue samples. These techniques were the basis for the development of OMICS technologies for the spatial mapping of analytes such as RNA, protein, and metabolites in their original position without disrupting the tissue architecture.

4.1. Microarray technologies

DNA microarray or DNA chip or biochip is a collection of microscopic DNA spots attached to a solid surface. Microarray techniques can be used for understanding conformational changes undergone in

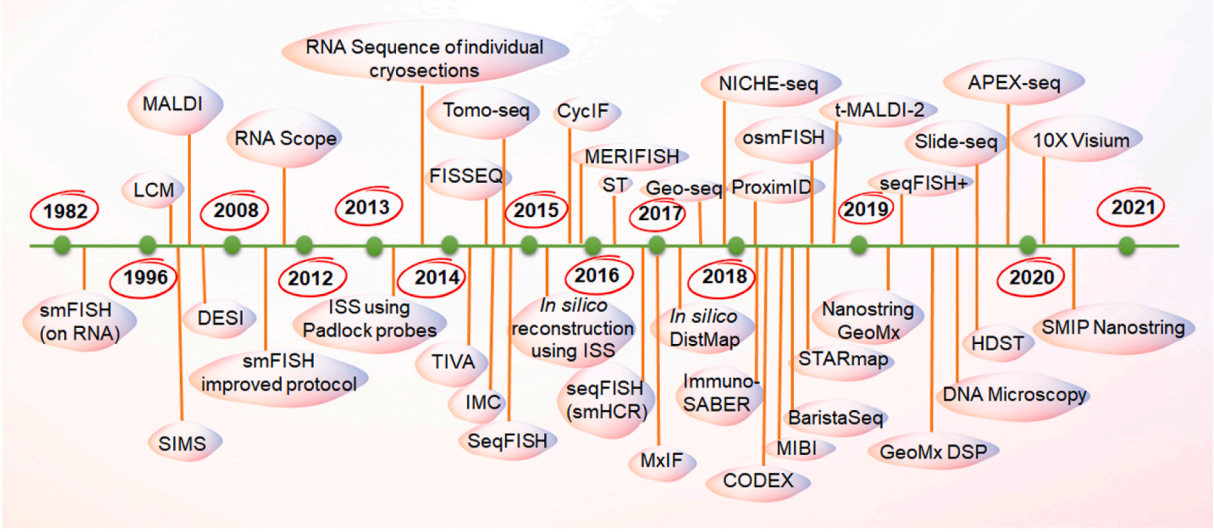

Fig. 2. A brief timeline of the spatial mapping performed with OMICS technologies used for molecular analysis of tissue samples. RNA tomography (tomo-seq), spatial transcriptomics (ST), cyclic-ouroboros single-molecule fluorescent *in situ* hybridization (osmFISH).

Table 1
High-resolution OMICS approaches used for the analysis of cancer tissue samples.

Technology	Applications	Strengths	Limitations/constraints	Refs
Multiplexed Error-Robust Fluorescent *in situ* Hybridization (MERFISH)	Transcriptome scale analysis of single cells in tissue samples	10,000+ mRNA species; resolution 1 μm. Suitable for both individual cells and tissue samples	high Host of the equipment and intensive training needed. Length of time for analysis (2–3 days per slide)	[29,34,42]
Slide-seq and 'spatial transcriptomics'	Spatial localization of mRNA within tissues section	Spatial profiling of mRNA with genome-wide, unbiased, high coverage capability	Specific oligomers are required, Slide-seq has resolved limited 'spatial transcriptomics' issues	[43]
Multi-region sequencing	Profiling the entities in heterogeneous tumor tissues	Maintain spatial resolution	Spatial resolution is extremely low	[44,45]
Laser capture microdissection (LCM)	Analysis of heterogeneity of cell populations in tissue section	Spatial information in native context and cell state retained	High-throughput analysis require automation	[46,47]
Matrix-Assisted Laser Desorption-Ionization Imaging Mass Spectrometry (MALDI-MSI)	Suitable for revealing spatial metabolomics and proteomics	Retains spatial information; label free technique	Semiquantitative	[48–50]
Secondary ion Mass Spectrometry (SIMS)	Lipids, metabolites, elements, and single-cell	Minimal sample pretreatment and complementary to MALDI, resolution power < 1 μm	Limited mass range, vacuum method, and matrix interference	[51]
Desorption electrospray ionization mass spectrometric imaging (DESI-MSI)	Lipids and metabolites	Minimal sample pretreatment, ambient method, and high sensitivity; 35 μm	Limited mass range, sensitive to surface, ill-defined sampling area and analyte washing effect	[52–54]
Laser ablation/inductively coupled plasma mass spectrometry (LA-ICP-MS)	Metabolites, metals	Minimal sample pretreatment and ambient method, 1 μm	Limited mass range and water-rich target needed	[55,56]
Imaging mass cytometry	Analyzing proteomic information of single cells	Single-cell protein quantification	Can perform up to ~50 parameters per cell	[25,57–61]
Cyclic Immunofluorescence (CycIF)	Profiling multiple of analytes in tissue samples	Useful for the analysis of a large number of proteins (~60) within tissues	Integrity of sample restricts the number of cycles; compatible antibodies limit the precise quantification	[62–64]
Histocytometry	Analysis of metabolites in single cells for *in situ* applications	Retains spatial information of analyte	Limited by specificity of antibodies	[65]
RNAscope	Profiling of *in situ* RNA molecules in intact tissue samples	The detection limit of RNA is up to 12 RNA copies	High cost of the equipment and intensive training needed	[66,67]
Digital spatial profiling (DSP)	Intra-tumoral heterogeneity in tissue samples	96 proteins and 1400+ RNA; resolution power 10 μm	Expensive equipment and time-consuming (10–20 slides/48 h)	[68,69]
Visium	Understanding the molecular heterogeneity of cells in tissues	Visium is potentially much higher throughput and less time consuming (Turnaround time is 4 capture areas/slide/day), Resolution 55 μm	High cost of the reagents	[70]

chromatin material (genomics), and epigenetic modifications, regulation of transcription, regulation of protein expression or metabolites [18,71,72]. Microarray-based platforms are considered high throughput approaches suitable for molecular analysis of biomolecules within tissue sections because of their uniqueness in performing the analysis of thousands of molecules within a single step [73]. For instance, high-definition spatial transcriptomics (HDST) microarray technology can determine the spatial distribution of RNA analytes in biological tissues with higher resolution than classical technologies by visualizing several thousand transcript-coupled spatial barcodes in a 2-μm tissue area within the mouse brain and breast cancer tissue samples [74].

Among OMICS technologies, DNA microscopy was used for spatial imaging of genomic-analyte in intact tissue samples [75]. The technique involved tagging the transcript with unique randomized nucleotides followed by an amplification reaction and then the application of a computational algorithm for locating the expressions of the gene of interest in their native tissue microenvironment [75]. The uniqueness of DNA microscopy is the ability to process many samples simultaneously and offer a new way for spatial profiling the biomolecular entities in tissues [8]. Furthermore, advances in super-resolution microscopy techniques such as fluorescence microscopy and electron microscopy enabled researchers to visualize the spatial maps of the genetic material at much higher resolution during the examination of living and fixed tissue samples as elaborated by Xu and Liu, 2019 in their recently published review article [76]. The data shows that electron microscopy techniques can visualize chromatin structures down to the limit of 20–30 nm which is equal to the size of packaged clusters of nucleosomes inside the nucleus [77].

Stahl et al. used a set of primers with positional barcode arrays that were fixed on the glass slide as shown in Fig. 3 . After this, tissue sections were fixed over the glass slides, then tissue was allowed to permeabilize, cDNA synthesis was performed, and later tissue debris was removed. The imaging of cDNA revealed the high quality of two-dimensional data of transcriptome in their positional context and thus revealing the spatial information of mRNA in mouse brain and human breast cancer tissue samples [24]. Moncada et al. used an oligo-deoxythymidine microarray approach to perform unbiased spatial mapping of transcriptome data within pancreatic ductal adenocarcinoma [78]. The uniqueness of this study was that it facilitated the differential imaging of multiple cells which helped to precisely detect cancerous parts of the tissue from a normal surrounding tissue sample. Similarly, another study was performed which also used the spatial transcriptomics (ST) technology to profile RNA analytes in tissue samples. In this work, ST/Visium array was used to fix thin slices of tissues, and RNA expression was recorded by barcoded capture-probes followed by sequencing using next-generation sequencing (NGS) methods. The sequencing and image data were processed by STUtility followed by image processing and data analysis carried out through the R package. This study provided a useful 3D map of mRNA expression by the application of STUtility software and image data processed by 10× Genomics Visium Platform [79]. Spatial profiling of formalin-fixed paraffin-embedded (FFPE) tissue was reported for the first time by using barcoded oligo(dt) probes in order to determine the mRNA expression in mouse brain and ovarian carcinoma tissue samples [80]. In addition, spatial transcriptomics technology can

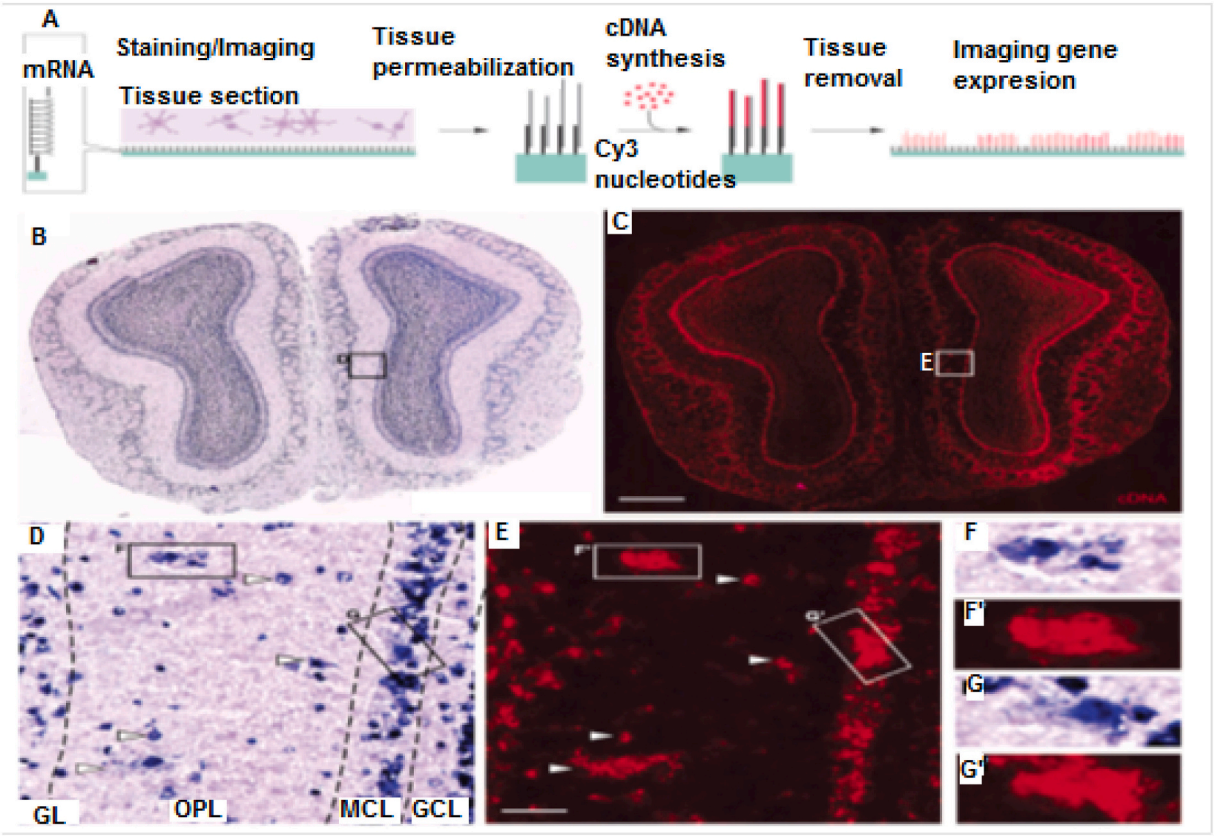

Fig. 3. Spatial profiling during cDNA synthesis. (A) the tissue sample is cryo-sectioned, fixed to the oligo(dT) primers and imaged. Removal of tissue shows fluorescently labeled cDNA synthesis incorporated with Cy3-labeled nucleotides. (B) Olfactory bulbs stained with H&E and (C) tissue removal displays fluorescently labeled cDNA. Scale bar, 500 mm. (D and E) Magnified portions (indicated by boxes) taken from (B) and (C). Cell layers and cDNA synthesis are highlighted with arrowheads and boxes. Scale bar, 40 mm. (F to G′) Magnified images in (D) and (E) displaying cytoplasm and cDNA content. Reproduced from Ref. [24] with the permission of The American Association for the Advancement of Science.

visualize whole transcriptome data and any number of genes within tissue section [81]. This was proved by the application of spatial transcriptome technology for analysis of mRNA expressions in prostate cancer [82] and melanoma tissue samples [83] by profiling of 6750 regions in the prostate and 2200 sites in melanoma. The results of this study displayed an existence of extreme heterogeneity in distinct gene expression signatures and the coexistence of unique expression profiles of different sets of genes in tissue biopsies [83]. This technique in combination with a machine learning algorithm for breast cancer was used by Yoosuf et al. to investigate the differences between invasive ductal carcinoma (IDC) and ductal carcinoma *in situ* (DCIS) [84] and was able to predict accuracy of 91% for IDC and 95% for DCIS. Berglund et al. used a spatial transcriptomic platform known as Agilent Bravo Automated Liquid Handling for profiling the mRNA abundances in tissue samples [85]. The platform was capable to perform a comparatively higher number of samples per run; sample preparation time is reduced by 35%, enormously reduces batch effects among samples and is highly accurate as well as largely free from technical mistakes during the sample preparation.

Another approach to study gene expression in the tissue section was adopted by Smela's group where they used a 2D-PCR reaction to amplify DNA from an 8 μm thick tissue and were able to amplify target DNA during the 35th cycle of the reaction [86]. Later, a 2D-RT-PCR reaction was adopted by the same group of researchers to visualize the low level of gene expression in intact tissue samples [87]. Recently, a similar study was performed that measured the spatial distribution of TOP2A mRNA in the prostate tumor tissue samples collected from mouse xenograft [88]. The tissue samples were fixed on the microchip for the analysis of TOP2A mRNA expression by real-time reverse transcriptase loop-mediated isothermal reaction (RT-LAMP). TOP2A mRNA expression in tumor tissue was confirmed by imaging the RT-LAMP reaction using fluorescent microscopy as shown in Fig. 4. TOP2A mRNA expression was carried out in 100 μm spatial resolution without the loss of its spatial location like intact tissue samples. The whole process from tissue loading to permeation and on-chip RT-LAMP reaction was completed within two hours. The approach is unique and has the potential for becoming a valuable technological platform for the assessment of spatial expression of analytes due to its fast turnaround, quantitative molecular outputs, and ease of use for pathologists and clinicians.

Polony (or DNA cluster)-indexed library-sequencing (PIXEL-seq) is another technology developed for spatial transcriptome profiling of tissue samples that uses “continuous” polony oligos arrays that are affixed over a gel surface. This technique can profile RNA transcripts within $\leq$1 μm resolution and can detect >1000 molecular analytes within 10 × 10 μm^2 tissue area. Therefore, this platform can perform transcriptome mapping below the subcellular level by maintaining high transcript detection efficiencies [40]. Geographical positional sequencing (Geo-seq) is another technique used to visualize the spatial localization and cellular heterogeneity of cancer tissues through combining LCM and single-cell RNA sequence technologies [89]. Another approach to study the spatial profiling was recently described by Ji et al. They have used scRNA-seq, spatial transcriptomics and multiplexed ion beam imaging technique to analyze the spatial localization of tumor-specific keratinocytes (TSK) within the breast carcinoma where they illustrated the existence of a population of TSK, immune infiltrates and heterogeneity along edges of tumor [90].

Although, it is well established that microarray technologies are high

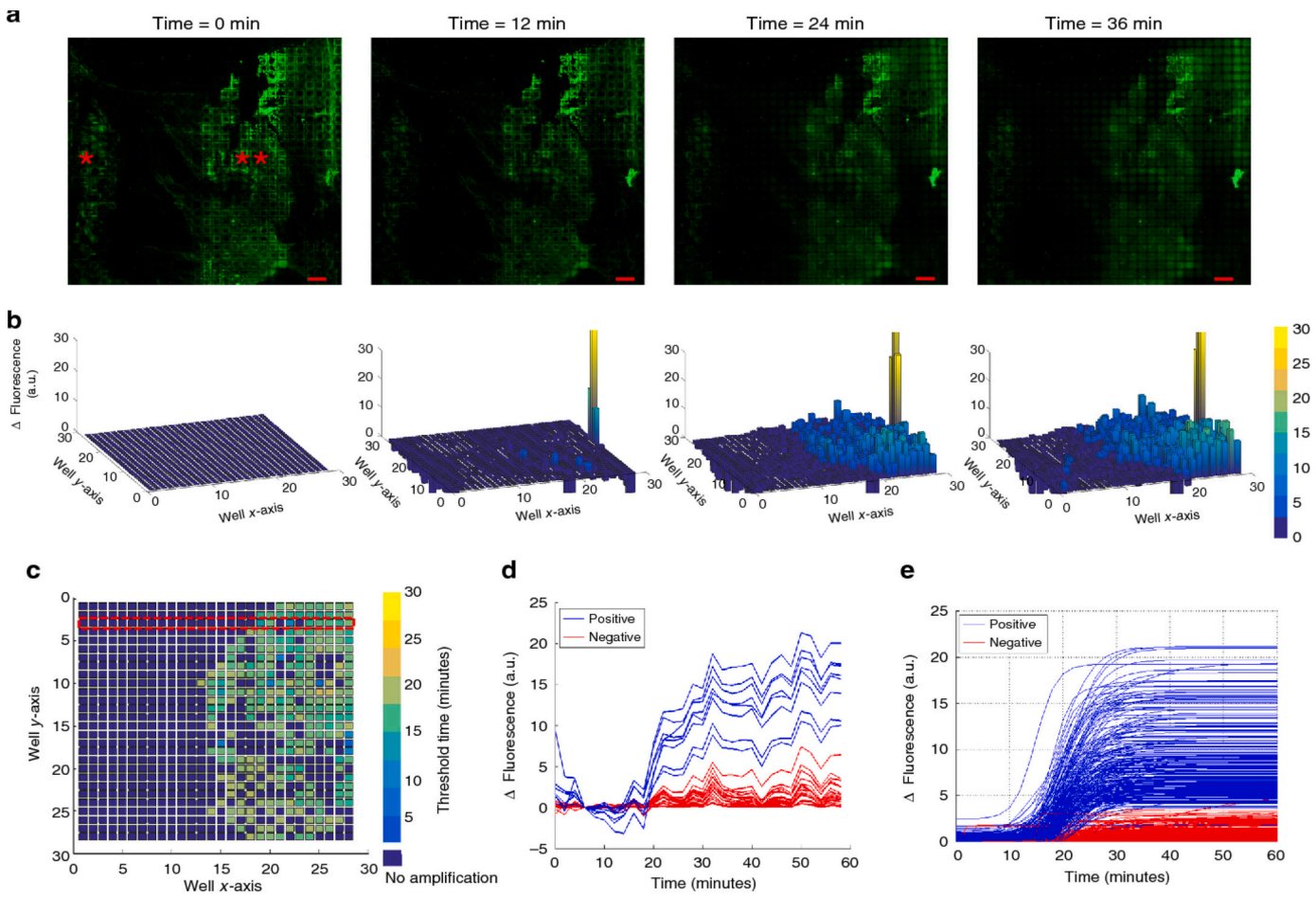

Fig. 4. RT-LAMP reaction performed on-chip: cancer *vs.* non-cancer control. *a* image of raw fluorescence displaying real-time RT-LAMP reaction with prostate tumor tissue on the right and noncancerous tissue on the left. *b* differential increase in fluorescence over time as displayed by bar graphs validate that amplification takes place in the cancerous tissue. *c* spatially mapped threshold times carried out for spatial threshold analysis where each pixel is 100 μm × 100 μm. *d* Raw amplification curves obtained from a row displaying positive and negative wells. *e* Fluorescence curves for all wells after curve fitting. Reproduced from Ref. [88] with Creative Commons Attribution-0.4 International License (CC-BY-0.4).

throughput and convenient methods for the analysis of molecular histology of tumor tissues by identifying tumor-specific signatures and discovery of potential therapeutics against neoplastic cells in prostate cancers [91]. The wide-scale applicability in spatial gene expression analysis is limited by several factors such as vast dynamic range, informational and experimental complexities and therefore, further advances are needed in microarray technologies for multiplexed analysis of tissue samples.

4.2. Mass spectrometry

4.2.1. Mass spectrometry-based spatial proteomics technologies

Mass spectrometry has been a common technique applied in the image analysis of the tissue sample for molecular profiling of elements and biomolecules. One of the unique features of MS-based technologies is that they can harness spatial information of analytes along with performing their quantitative analysis of tissue samples. In MS-approaches, the analysis of analytes is carried out in different ways such as fractionated organelles post-lysis method and protein-protein interaction network analysis approach [92]. Analysis of protein molecules can be performed in another way that involves the use of antibodies to do the purification of target analytes (called "baits") and binding partner molecules which are followed by the analysis with MS technique [92]. These techniques can do spatial proximity measurements and thus, fail to perform spatial localization of analyte protein molecules in single cells. Therefore, localization of proteins in their spatial context is performed with cytometry-based imaging approaches. The protein analytes are labeled with metal-labeled antibodies in intact tissue sections and then their spatial distribution is performed with imaging mass cytometry (IMC) [25] or multiplexed ion beam imaging (MIBI) [33]. During the analysis of protein molecules by the IMC method, tissue sections are transferred voxel-by-voxel into a cytometry by time-of-flight (CyTOF) mass cytometer which is followed by the analysis of the protein analytes through metal isotope content. While analysis of protein analytes from tissue samples by MIBI technique consists of scanning over the ion beam over tissue sample which leads to the generation of secondary ions containing the metal isotopes tagged with the antibodies. Currently, with the help of sector mass spectrometer, metal isotopes concentration and finally protein profiling is performed within tissue samples. Analysis of protein entities with cytometry-based imaging techniques is useful as these methods can perform profiling of molecules up to absolute spatial resolution level besides doing the multiplex imaging of target proteins (Fig. 5). However, these techniques can map analytes molecules up to 200 nm spatial resolution due to the limited precision of laser ablation and ion beam milling [93]. Secondly, the multiplexing capacity of both of these techniques is limited to 44-plex due to their dependence on metal-modified antibodies [93].

The spatial analysis of protein distribution within tissues has proved that there is a close association between protein functions and cellular activities [92]. Spatial mapping of proteins within tissues with the use of

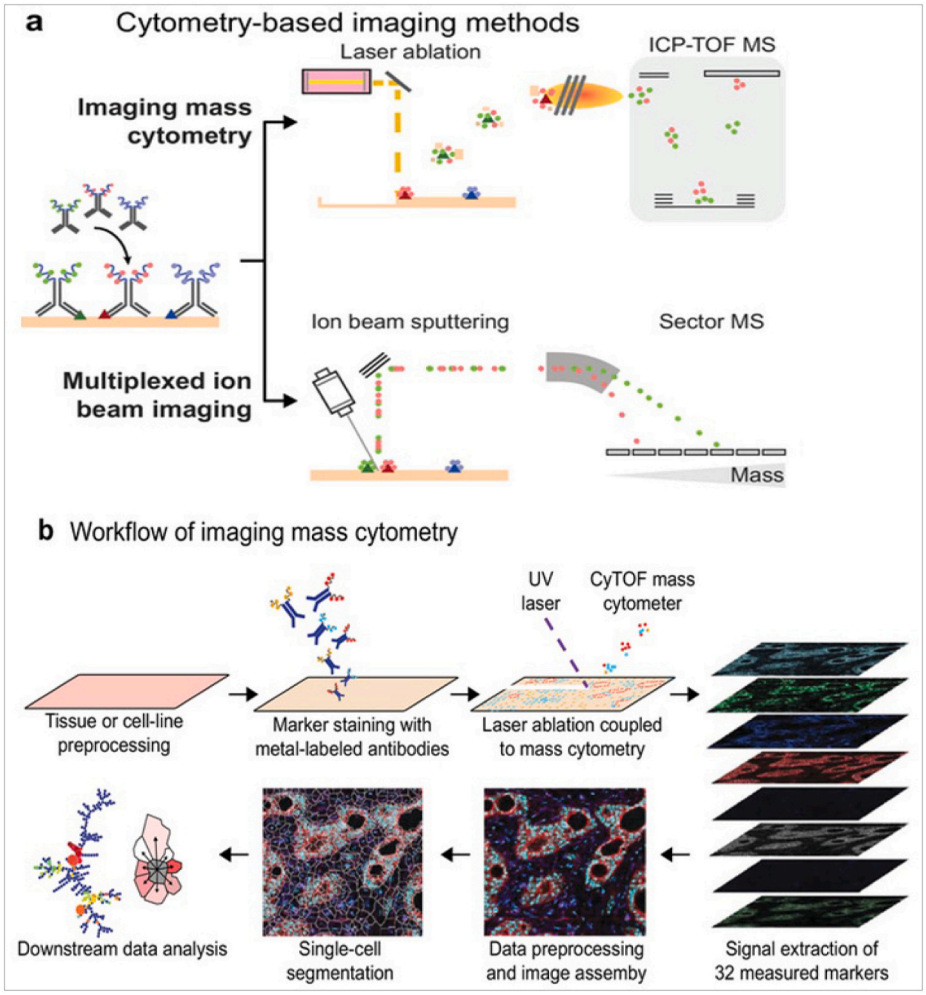

Fig. 5. Cytometry-based imaging methods where (a) during imaging mass cytometry, thin slices of tissues are stained with metal-labeled antibodies. Here, laser ablation carries out the sectioning of tissue and by the analysis with CyTOF-MS, the content of metal isotope is determined and thus epitope expression. While multiplexed ion beam imaging (MIBI) involves the use of a primary ion beam to generate secondary ions among metal isotopes that are attached to the antibodies. After this metal isotope content is determined by a sector field mass spectrometer and corresponding epitome expression is estimated. Reproduced from Ref. [93]. b) Steps performed during imaging mass cytometry. Tissue sections are labeled with metal-modified antibodies which is followed by ablation of tissue sections in a voxel-style and then analyzed with CyTOF mass cytometer. The spatial information of protein is collected by combining the ablation data with the CyTOF step used for the identification of proteins leading to the acquisition of multiplexed "image" of spatially distributed 44 proteins. Reproduced from Ref. [25].

mass spectrometry exhibits that dynamic changes occur in protein components especially at the subcellular level that varies among different cell types and their proliferative states. The analysis of protein components within tissues is carried out by label-free quantitation and stable isotope labeling-based quantitation methods [94]. Label-free mass spectrometry approaches perform relative quantifications of proteins and lipids in biological tissue samples as these techniques can profile analytes with spectral counting without the requirement of additional chemistries [95]. Label-free quantitation strategies do not require the labeling step of proteins or peptides during cell culture or sample preparation steps [96,97]. Besides, recent developments in instrument analytical capabilities and the use of algorithms in data handling have increased the value of label-free techniques for their simplicity and cost-effectiveness [98]. However, their-wide scale applicability is hampered by their limited multiplex capacity and quantitation accuracy [99]. On the other hand, analysis of tissue samples with stable isotope labeling-based methods is achieved by labeling the tissue samples with different stable isotopes, and then samples are run through the same LC-MS/MS [100]. As compared to label-free approaches, stable isotope labeling-based methods can profile analytes quantities from multiple samples (high multiplex capacity) simultaneously with better accuracy [101]. Additionally, stable isotope labeling-based quantitation methods have shown huge prospects for spatial mapping of protein contents by integrating these approaches with proximity labeling methods [102] or with subcellular fractionation [103,104]. Recently, there have been interesting developments in approaches that use tandem mass tag (TMT) reagent for spatial localization of protein analytes such as in TurboID [105], Localisation of Organelle Proteins by Isotope Tagging after Differential ultraCentrifugation (LOPITDC) [106], hyper-LOPIT [107], and SubCellBarCode [108] methods. These methods allow the analysis of protein contents at much higher resolution (up to 10 subcellular compartments), quantification of >5000 proteins with an accuracy of >90% [92].

In this context, desorption mass spectrometry and secondary ion mass spectrometry have been used extensively for the molecular analysis of analytes for over 20 years [109]. These techniques, however, were not found useful for the image analysis of proteins and polypeptides in the tissue samples. Among the mass spectrometry techniques, MALDI-MSI is capable to analyze the spatial localization of analytes within a spectral resolution of 1.4 μm which is the highest resolution of the MALDI-MSI technique [110]. This technique, thus, preserves the distribution of analytes in tissue samples in a spatial context. In an interesting study, the spatial distribution of erlotinib in the superficial layer of normal and skin tissue containing rashes was analyzed with MALDI-MSI. The results showed that the concentration of erlotinib is much higher in the affected skin layer compared to the normal skin layer [111]. It was observed that desorption electrospray ionization (DESI) and matrix-assisted laser desorption/ionization techniques rely on the desorption of ions from tissue surfaces for performing MSI [112–114]. DESI-MSI technology is a high-throughput technique, can perform tissue analysis with improved resolution up to a limit of 35 μm having a higher peak capacity and signal-to-noise data of tissue samples for analyte analysis [52,53]. Operating the DESI-MS in two dimensions over the tissue surfaces gives a more detailed biochemical analysis of analytes especially lipids and metabolites by recording mass spectral data pixel-by-pixel followed by plotting them into ion images

[113]. Analysis of prostate cancer tissue by DESI-MSI technique showed that this method is capable to analyze the distribution of glucose and citrate levels in prostate and normal tissue samples less than 1 min with 90% accuracy compared to the histopathological analysis used for frozen tissue sections [115] while profiling the distribution of Krebs cycle intermediates across prostate cancer tissues by DESI-MSI revealed that this technique can distinguish tumor tissue from normal tissue with 90% accuracy [116]. Impressed from the uses of DESI-MSI technology for its ability to analyze the issue samples within a range of 35 μm [53], more advanced mass spectrometry techniques in the form of MALDI-MSI were developed which were able to profile a large number of analytes inside tissue samples within a spatial range of ~20 μm. Fincher et al. showed that DESI-MSI can detect simultaneously a broad range of analytes in tissue sections by incorporating the silicon nanopost array (NAPA) within tissue sections [117]. The technique can perform selective ionization of triglycerides (TGs) by laser desorption ionization technique (LDI) and result in an increase of ~105 and ~ 49-fold signal intensity for TGs in the mouse lung and human skin tissue samples. In this way, MSI in combination with NAPA can visualize neutral lipids such as triglycerides (TGs) which are normally suppressed by phospholipids; thus, analysis of these vital signatures practically becomes impossible. Rompp et al. showed that among different MS technologies, AP-MALDI-Orbitrap and TOF-SIMS technologies show improved resolution even at a very low-micrometer range compared to other MS techniques [118]. Among the available mass spectrometry methods, MALDI-MSI can perform non-targeted analysis of protein molecules by visualizing hundreds of analytes each time as described in a recently published review article [119]. In addition, another mass spectrometry technique named as matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry imaging (MALDI-FT-ICR MSI) can profile protein distribution among the tissue samples collected from high-grade glioma of a mouse model. The advantage of the use of FTICR in this study is that it overcame the challenges of overlapping along with isobaric distributions of specific protein analytes during the imaging process [120]. MALDI-FT-ICR-MSI was tested on FFPE tissue samples for analysis of metabolites content displaying a detection limit of ~1500 *m/z* species and mass range of m/z 50–1000 from FFPE tissue samples. Analysis of tissue samples using this platform is quite advantageous as it can be completed with 24 h depending upon the size of the tissue sample to be measured and the raster size used. Besides sample handling with this method is quite easier, has a higher throughput and reproducibility, and can perform molecular spatial distributions *in situ* studies [121]. MSI techniques were used to map the metabolites in the tumor tissues as it is a proven fact that cancer cells reprogram their metabolism to modulate their cell proliferation, cell migration and cell differentiation potential in cancerous tissues [122]. Spatial analysis of tissue samples from esophageal cancer patients with airflow-assisted desorption electrospray ionization mass spectrometry imaging (AFADESI-MSI) exhibited that there has taken place metabolite reprogramming in the tumor-associated metabolic pathways of glutamine metabolism, proline biosynthesis, uridine metabolism, histidine metabolism, fatty acid biosynthesis, and polyamine biosynthesis. Furthermore, it was established that alternation in metabolic pathways has been a result of abnormal expression of metabolic enzymes such as pyrroline-5-carboxylate reductase 2 (PYCR2) and uridine phosphorylase 1 (UPase1) in esophageal squamous cell carcinoma (ESCC). In another study, Sun et al. used the MSI technique for the spatial localization of carnitines reprogramming in breast cancer tissues [123]. The study involved the spatial mapping of carnitines enzymes and their associated metabolites in the tissue sections of mouse models. The results obtained by MSI-based analysis of normal and tumor tissues showed that carnitine palmitoyltransferase 1A (CPT 1A), carnitine palmitoyltransferase 2 (CPT 2), and carnitine acetyltransferase (CRAT) are abnormally expressed in the cancer tissues. Thus, it was concluded that carnitines metabolism is reprogrammed in cancer tissues which can be used for the identification and diagnosis of tumor tissues. In addition, the role of metabolite reprogramming was analyzed by MSI-based spatial mapping of metabolites in thyroid cancer tissue samples [22]. The study revealed that tissue heterogeneity is linked with metabolic reprogramming in the thyroid tissues. Thus, it could be concluded that spatially resolved metabolomics provides insights for understanding the alternations in metabolic enzymes and their target substrates which has been useful in improving knowledge of metabolic reprogramming.

Protein expression patterns profiling carried out by quantitative mass spectrometry (qMS) explored the role of biomolecules in the development of tumor heterogeneity. High-resolution qMS enabled researchers to profile proteins contents with much better spatial resolution and coverage area with the ability of generating reproducible results. For instance, in one of the studies in this direction, Buczak et al. profiled the spatial distribution of thousands of proteins in hepatocellular carcinoma with qMS and found that several proteins which are associated with the tumor development are heterogeneously expressed in the tissue samples [124]. Boisvert et al. used the qMS technique to determine the spatial distribution of proteins within HeLa cells and were able to quantify a total of 80,098 of peptides from 8041 proteins which displayed their spatial distribution inside the cytoplasm, nucleus, and nucleolus by the analysis of data with PepTracker software. Pressure cycling technology (PCT) and Sequential Windowed Acquisition of all THeoretical fragment ion mass spectra (SWATH) have been useful for determining the intratissue heterogeneity (ITH) of prostate cancers by profiling protein abundances in these tissue samples [125]. Results obtained with PCT showed that there are around 6873 different types of proteins involved in the development of ITH in prostate cancer patients. The spatial organization of metals in pig melanoma tissue was assessed by mapping the adjacent tissue sections by a combination of mass-spectrometry and histology through the image registration method [126]. The technique involved the collection of digital data from the region of interest of tissue section as a histological scan and its adjacent neighboring region by laser ablation inductively coupled plasma mass spectrometry. The data was then processed to find the distribution of metals in spatially at the selected histological zones. Application of hematoxylin-eosin staining, and laser ablation inductively coupled plasma mass spectrometry showed a significant increase in the concentration of Cu and Zn cryosections of Melanoma-bearing-Libechov-Minipig (MeLiM). In another study, MasSpec Pen methods were used for the real-time analysis of analytes such as proteins, lipids and metabolites in tissue samples [127]. The use of statistical tools such as least absolute shrinkage and selector operator (Lasso) in MasSpec Pen technology showed that it is highly sensitive and specific for the analysis of specific analytes [128]. Nanodroplet Processing in One pot for Trace Samples (nanoPOTS) was developed for the profiling of proteomic samples [129]. The platform can reduce the total volume of sample from hundreds of microliters to <200 nL using a single droplet reactor. This platform termed nanoPOTS in combination with LC-MS can quantify 1500–3000 proteins only from 10 to 140 cells which were possible previously from thousands of cells only. In addition, analysis of 10-μm-thick pancreatic tissue sections with nanoPOTS demonstrated that this platform can quantify ~2400 proteins from single human pancreatic islet cross-sections which have profound importance for understanding tissue cellular heterogeneity in type 1 or type 2 diabetes. The further details of the use of MSI technologies for the understanding of biological and pathophysiological processes in the context of various analytes in their native environment was reported by Siegel et al. in their recently published review article [130].

4.2.2. *Antibody-based spatial proteomics technologies*

Multiplex immunohistochemistry/immunofluorescence (mIHC/IF) is used for the simultaneous detection of up to 40 entities/targets in a tissue sample [131]. This can help researchers for profiling the spatial distribution of analytes and thus understand the tumor microenvironment. However, this technique can perform multiplex spatial analyses of only a limited number of molecules during a single measurement

compared to other genomic techniques [32]. Co-detection by indexing (CODEX) is another imaging platform that can simultaneously detect up to 60 markers during *in situ* analysis of cancer tissue sections [132]. The technique involves the use of DNA barcode strands which are hybridized with complementary "primer" strands. DNA polymerase along with a single dye-labeled nucleotide during the first-round performs the extension of primer on the target protein which is to be imaged. The extension of the DNA barcode on the protein species that has to be imaged is carried out by the nucleotide during the second round. After round one, the fluorophore is removed. Thus, CODEX is capable of mapping up to 60 markers in cancer tissues [132] and 22 different proteins of interest in mouse-isolated splenocyte cells [133]. The drawback of these techniques is that they can scan to a 4 × 4 mm area thus unable to visualize analytes in broader areas of larger tissues. Secondly, former techniques need fixation of tissue sections over slides as a pre-processing step. Another technology named as Multi-Omyx can perform spatial mapping of tissue samples by staining the tissue samples with fluorescent antibodies followed by consecutive imaging and thus non-destructive chemical quenching of fluorophores. The images recorded in this way are stacked together and spatial analysis up to a single cell level is carried out with an algorithm [134]. Multi-Omyx technology can analyze over 60 analytes and is considered very useful for analyzing FFPE samples. The advantage of using Multi-Omyx technology is that it performs analysis of tissue samples using customized targets. However, when multiple cycles are used for imaging of tissue sections, loss of immune affinity may hamper analyte analysis and therefore, antibody combinations are validated for sequential application.

4.3. *In situ hybridization (ISH)*

Single-molecule fluorescence *in situ* hybridization (smFISH) [1,2] is a useful approach for profiling the expression of individual genes in their original positions within a cell. SmFISH technique is extremely sensitive and specific as it could quantify a single mRNA molecule from tissue samples. The technique involved the hybridization of multiple sets of fluorescently labeled oligonucleotides to a single mRNA molecule at different sites which lead to an increase in signal to noise and thus result in improved accuracy in detecting mRNA molecules of interest [2]. The quantification limit of mRNA copy number was improved with 10plex detection that used different fluorophores and combinatorial labeling with spectral barcodes of two colors [135]. Later, detection of a single mRNA molecule was further improved by the use of smFISH with combinatorial labeling and super-resolution imaging that was designed to prevent the overlapping of mRNA spatial and spectral barcodes for *in situ* applications [136]. The expression value of genes in a cell can be determined by measuring the intensity of the fluorescent signals. Sequential Fluorescent *in situ* Hybridization (SeqFISH) had been used for performing multiplexed smFISH together with combinatorial labeling for the precise analysis of mRNAs [137,138]. Multiplexed error robust FISH (MERFISH) is another advanced platform that uses the two-step labeling approach and combinatorial labeling for detection of the mRNA molecule even to a single copy number [139]. The method consists of hybridization of specific encoding probes composed of complementary sequences and two flanking readout sequences. The approach detects the analyte through several rounds of hybridization only within 15-min compared to contemporary methods which require usually more than 10 h for profiling mRNA expression. One of the main disadvantages of this technique was that the rate of detection of mRNA increased exponentially with the increase in the hybridization rounds [139]. This drawback was reduced using a modified Hamming distance error correction code with a minimum distance of four and a constant number of four 1 bit per RNA binary barcode (since 1 to 0 errors are the most common for smFISH). Furthermore, the application of chemical cleavage of fluorophores and thus, multicolor imaging resulted in the decrease of hybridization rounds and thus imaging time was substantially decreased in MERFISH [140].

In another interesting study, an attempt was made to determine the effect of mutations in tumor cells by comparing the single locus or set of loci of tumor DNA and germline DNA through amplification of chromosomal regions by FISH and then sequencing specific genes [141]. The approach involved in this technique consisted of the use of FISH and immunofluorescence for determining the genetic and phenotypic diversity of various kinds of cells. The study involved the use of image cytometry combined with multiscale microscopy images and the acquisition of single-cell data through image processing. The method allowed demonstrating the effect of metabolites on the tumor-associated macrophages (TAMs) in the tumor microenvironment to differentiate into specific types of cells depending upon the local levels of ischemia and their position relative to the vasculature.

Advances in the fourth generation RNAseq platforms especially *in situ* sequencing (ISS) and fluorescent ISS (FISSEQ) have greatly helped to achieve the objectives and aims of RNAseq technologies [142,143]. ISS technique involves the use of padlock probes and rolling circle amplification (RCA) to generate targeted sequencing libraries sequenced by NGS technologies [14]. ISS technology can sequence up to 256 copies of RNA in a single step, therefore, it is regarded as a high multiplexed technique for the analysis of RNA transcripts, however, the assay throughput of this platform is low, and the method is rather time-consuming. Fluorescent *in situ* sequencing (FISSEQ) on the other hand is similar to the ISS platform and can perform *in situ* imaging for multiple rounds to read out the sequence of RNA analyte [14]. Studies showed that the FISSEQ technique compared to ISS could produce random libraries, unbiased RNA analysis and could detect even very low RNA copy numbers during the analysis of single-cells [144]. However, as far as sensitivity to RNA was concerned, the ISS technique was two times more sensitive as compared to FISSEQ methods [145]. Both ISS and FISSEQ technologies have been commercialized by two companies Cartana and ReadCoor, respectively, and these two companies are now part of 10× Genomics. Recently, a novel technology named Slide-seqV2 has been developed which is more sensitive to Slide-seq technology showing an enhanced RNA capture efficiency ~50% to that of single-cell RNA-seq data during the spatiotemporal analysis of RNA expressions in the neurons of mouse hippocampus. This technology has 10-fold higher RNA detection efficacy which is near to the detection efficiency of droplet-based single-cell RNA-seq techniques [37]. Another study published recently describe the use of deterministic barcoding in tissue for spatial omics sequencing (DBiT-seq) platform for constructing multi-omics atlas using fixed frozen tissue sections [38]. The platform involves the use of DNA oligo barcodes that are incorporated into lysed tissue samples by a microfluidic device. The developed technique has the potential of performing spatial analysis of transcriptome up to single-cell and spatial distribution from a panel of proteins. In the context of spatial barcoding technologies, Seq-Scope was used for exploring the transcriptomic profiles of histopathological tissue samples such as liver and colon [39]. The technique involved the solid-phase amplification of randomly barcoded single-molecule oligonucleotides and subsequent formation of RNA-capturing barcoded clusters that are visualized to be ~0.5–0.8 μm apart from each other. Thus, Seq-Scope can perform spatial transcriptome analysis quickly and accurately using a straightforward protocol that is easy-to-implement for the analysis of tissue samples. Spatial Transcriptomics by Reoriented Projections and sequencing (STRP-seq) was recently applied for the analysis of transcriptome data by combining compressive sampling and image reconstruction approach to the mouse brain tissue sections [7]. The method presented here involved the use of tissues which were sliced into 14 μm thick primary sections and then these were used to produce secondary sections named (tissue strips) by cutting them at various angles. To prepare a library of targeted transcripts and generate the raw data, lysis of tissue strips and RNA extraction was performed that was followed by RT-PCR reaction, tagmentation and sequencing. Finally, analysis of transcripts expression was carried out with the Tomographer

algorithm. Thus, a platform developed using these steps enabled the team to produce spatial profiles of RNA transcripts by transforming the low-input RNA sequencing technique into an advanced imaging-free spatial transcriptomics method that possessed the capacity to profile the transcriptome of the murine brain. Another platform developed recently reports the application of Spatiotemporal Enhanced REsolution Omics-sequencing (STERO-seq) which can perform spatial analysis of transcriptome at nanoscale level using DNA nanoball (DNB) patterned over array chips for *in situ* detection of RNA inside tissue samples obtained from mouse brain. The method allows spatial transcriptome analysis with high throughput within centimeter-scale areas displaying high sensitivity and homogenous RNA capture rate [146].

Further details for the use of analytic and experimental technologies in the context of single-cell multi-omics evolved in recent years can be studied from a recently published review article by Nam et al. [147]. This work comprehensively describes the capture potential and integration of multiple data modalities that are highly important for understanding the mechanism involved in cancer evolution. Furthermore, multiplexed imaging technologies open new directions in the field of pathology of cancer tissues. Spatially resolved proteomic, genomic, and metabolic profiles of human cancers are now possible at the single-cell level. This perspective discusses spatial bioimaging methods to decipher the cascade of microenvironments in solid and liquid biopsies [148].

4.4. Digital pathology and machine learning

DSP can perform high-plex spatial mapping of analytes such as RNA and proteins by the application of oligonucleotide detection technologies. The platform involves the use of oligonucleotide barcodes which are conjugated with the antibodies with a linker that is light sensitive and can be removed with UV light exposure. High plex oligo tags are removed with the exposure of UV light and then their quantitative analysis is performed. After this, oligo barcodes are mapped back to tissue location in order to perform spatial analysis of molecules in the area of interest. DSP technology is high-throughput as it can perform the analysis of 16–20 tissue sections per day [32]. Analysis of tissue samples from melanoma patients using the DSP platform revealed that there is a correlation between baseline immune infiltration and treatment response [149,150]. Recent advances in the field of DSP have enabled researchers to perform spatial localization of genes in an accurate and high-throughput manner. In this direction, nCounter R barcoding technology developed by NanoString Technologies can spatially map the expression of up to 1000 mRNA by a highly multiplexed assay [151]. GeoMx DSP platform pioneered by NanoString technologies use selected gene panels for mapping of RNA and protein analytes to explore gene expression patterns for better understanding of tumor microenvironments and immuno-oncology [152]. In the context of DSP, Ihle et al. [153] studied the tumor microenvironment of the bone tissues in prostate cancer patients and found that there is a distinct population of immune cells in the lytic and blastic lesions of prostate cancer. DSP showed that the expression of genes involved in the pSTAT3 and JAK-STAT pathway was quite enriched in blastic lesions while in lytic lesions, the expression of genes related to the pAKT activity and PI3K-AKT pathways was tremendously pronounced. DSP technologies have found wide-scale applicability in biomarker discoveries and optimizing therapeutic strategies [149,153]. The results further showed that expression of high or intermediate mRNA of IFN-γ biomarker after a combinatorial therapy with ipilimumab and nivolumab did not experience a relapse of melanoma while low expression of mRNA of IFN-γ signature showed a higher relapse in high-risk melanoma patients [153]. The DSP studies thus helped in the identification of analytes involved in cancer progression and opened the possibilities for finding the best immunotherapy strategies.

There has been tremendous growth in the number of high throughputs multiplexed platforms due to robust advances taking place in high throughput sequencing, live DNA imaging, and genome engineering techniques. This has led to do spatial analysis of thousands of analytes such as RNA and protein molecules without homogenization of tissues. One example in this context is of Visium Spatial Gene Expression which has gained importance for its high throughput and high sensitivity properties. This platform is owned by 10× Genomics and can perform digital spatial profiling of RNA molecules by automated labeling of tens of thousands of analytes of both fresh tissue sections and standard tissue blocks. Visium Spatial Gene Expression, therefore, can profile whole transcriptome high resolution across thousands of areas in tissue section ranging from 1 to 10 cells per spot. Thus, this platform is important for determining the gene expression patterns, the spatial distribution of specific biomarkers and tissue histology based on transcriptomic profiles [154]. 10× genomics has recently acquired CARTANA which is another platform developed by a Swedish spatial genomics company. CARTANA has developed reagents such as gene panels and *in situ* sequencing kits that can perform analysis of more than 100 genes at single-cell level using *in situ* sequencing technology. This technology is good for spatial mapping of analytes/genes from both fresh/fixed frozen or FFPE samples which can increase our understanding of different disorders such as inflammation, neurodegenerative diseases, and cancer [155].

Digital pathology, machine learning and artificial intelligence platforms have provided fast and accurate analysis of WSIs by the application of computational algorithms and annotations. In this direction, an interesting study was reported that the use of digital and machine learning approaches focused on detection of early neoplastic lesions in the prostate cancer tissue samples collected from the mice model [156]. FFPE and H&*E*-stained prostate and tissue samples were imaged by microscopy and the demarcation of the region of interest was performed. Further processing revealed that tumor genotypes have a close association with the tumor pathological characteristics which has a huge significance in the determination of tumor aggressiveness and resultant drug response. The technique provided a new way for evaluating spatial gene expressions and the way of classifying the tissue samples belonging to diverse histology and different genetic backgrounds with high specificity and sensitivity [157]. In another interesting study, Schmauch et al. applied DSP for the localization of RNA sequences from WSLs using a deep learning module referred to as HE2RNA [158]. The details of the application of the HE2RNA module are displayed in Fig. 6.

In the context of digital pathology, an interesting study was performed to assess the spatial mapping of tumor-infiltrating lymphocytes (TILs) from H&E images of 13 The Cancer Genome Atlas (TCGA) tumor types [159]. Digital localization of TIL from H&E images was carried out through computational staining by training of convolutional neural networks. The results showed that images of tissue samples were enriched with T cell subpopulations and TIL densities and spatial distribution in different tissue types reflect tumor cell aberrations. In another study, DSP was applied to study the location of RNA and proteins with the use of oligonucleotide detection technologies in colorectal and lymphoid tissue samples [160]. The platform was able to map 40 proteins and over 900 mRNA molecules showing unlimited multiplexing capability for the analysis of heterogeneous tissue samples. Furthermore, a recently published work reported that the application of machine learning algorithms such as ST-Net by combining spatial transcriptomics data and image data could profile mRNA expression in heterogeneous breast cancer tissue samples [161]. The technique was remarkable as it showed a resolution of 100 μm and could predict the spatial variation of 102 genes across breast cancer tissue. Bergenstrahle et al. reported the use of an R package named as SpatialCPie which was used to combine the cluster of transcriptomics data obtained at multiple resolutions from tissue samples of developing heart, breast cancer and melanoma patients [162].

Another distinction of DSP is that it can carry out digital quantification of analytes by the application of the bead-on-string barcoded counting method and thus improves the throughput of 12 samples for 90-plex protein assays in three days. The results obtained by DSP have

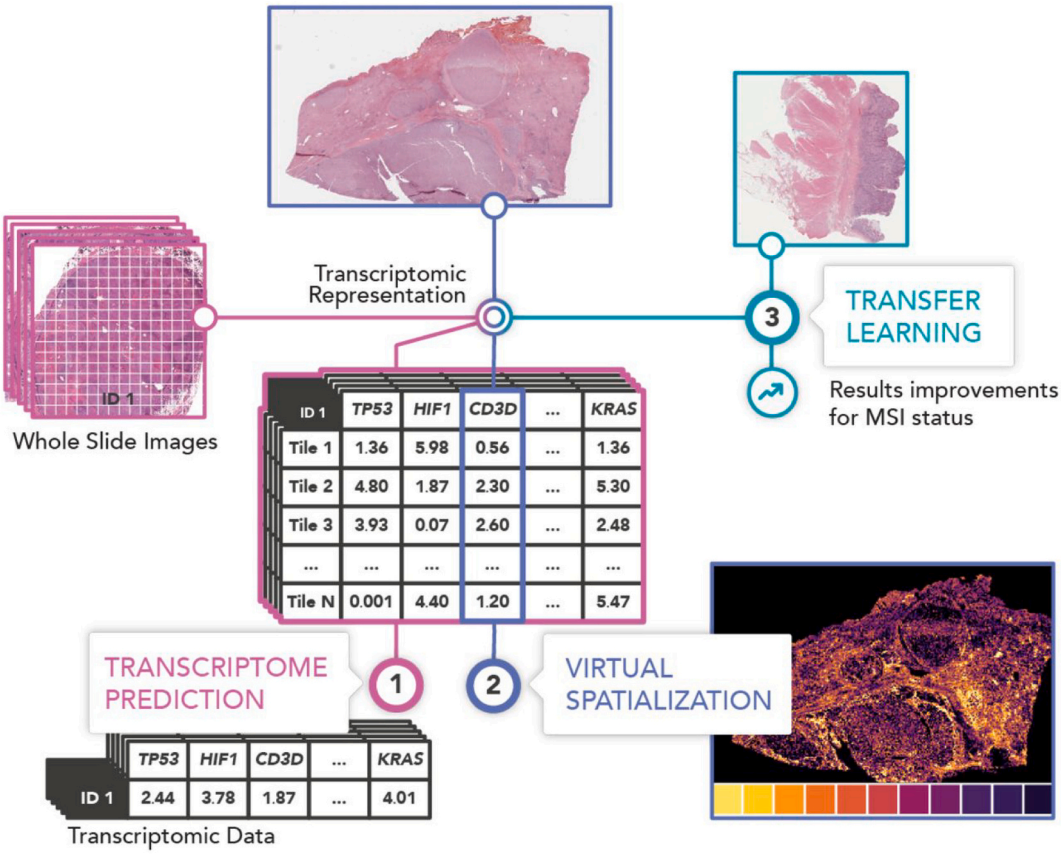

Fig. 6. H&E-stained tissue slides and RNA-Seq data was obtained from The Cancer Genome Atlas (TCGA). Transcriptomic profiles of whole slide images (WSIs) were collected after training with neural network HE2RNA. Neural networks extracted the information by internal learning through t tiled images and gene expression levels. The transcriptomic profiling can be helpful for 1. Transcriptome analysis without related RNA sequencing from WSI. 2. Transcriptomic data profiling by virtual spatialization. Each tile of WSI is used to predict scores for coding or non-coding genes which are later used in the form of heatmaps to predict the expression of the corresponding gene. 3. Enhancing predictive power transfer learning tasks such as microsatellite instability (MSI) status prediction from non-annotated WSIs. Reproduced from Ref. [158] with the permission of Springer Nature.

been confirmed and validated using IHC and quantitative immunofluorescence [32]. Although, the study was not originally designed for addressing the questions of RNA and protein quantification at a single-cell level, its ability to quantify mRNA and proteins at sub-population allowed researchers to analyze tumor microenvironment [32]. The ultra-sensitivity of this technique was found suitable for the quantification of entities from 5 cells with outstanding linearity and helped the researchers to obtain TLSs spatial expression patterns in melanoma cells [163]. NanoString nCounter and GeoMX platforms were used to assess the spatial distribution of transcripts and proteins in pancreatic cancer patients. This allowed differentiating tumor cells from immune cells, and stroma regions among patients who were receiving different therapeutic combinations. The spatial distribution of markers thus was useful for the identification of therapeutic related expressions and further studies for the investigation of biomarkers [164]. Analysis of biopsy samples from pre and post-treated uveal melanoma patients revealed that there arise some treatment-specific immune-alteration in different patients [165].

5. Opportunities and prospects

Traditional immunohistochemistry or *in situ* hybridization techniques rely on probing protein and/or nucleic acids of a relatively small number of known targets to examine gene expression profiles within tissues. This requires apriori knowledge of protein biomarkers but due to limited studies of known targets, researchers may miss the information about protein or cell subtypes that could be biologically significant. Examining a wide range of biomarkers with classical pathological techniques requires many sections, probed with targets individually or with low-plex throughput, and finally, all the sections need to be imaged and manually analyzed. In cases where the amount of tissue available is limited, analyzing extensive targets may not even be possible. The application of advanced OMICS technologies could create a wealth of information that can be gained from a single tissue section, which is particularly advantageous for studies where the amount of tissue is limited. Furthermore, exploring gene expression patterns in an unbiased manner with spatial gene expression profiling deepens our understanding of the factors that contribute to normal development and function, as well as mechanisms that underlie cancer. For instance, the use of scRNA-seq technologies will reduce unbiased cell classification for the identification of exact types of cells in different types of human tissue samples. In this context, the contribution made by the international consortium termed “the Human Cell Atlas” will be useful for spatial information to generate comprehensive reference maps for the diagnosis and treatment of complex diseases. The spatial information collected by single-cell analysis will have a significant impact in the field of tissue engineering and precision medicine. Moreover, recent discoveries that combine imaging and molecular profiles to train machine-learning algorithms for image-based cell classification and *in silico* diagnostics will be the benchmark for spatial profiling of tissues in their 2D and 3D contexts.

Recent advances and discoveries in the fields of cellular imaging and molecular analysis have opened a new window for spatial mapping of cells within tissue environments but there are a few challenges such as

the application of these technologies on the same cell in tandem. The use of laser particles as multiplexed imaging probes will be able to fill the gap for precise detection of an analyte with improved resolution; thus, will enable bridging of different technologies to perform massively multiplexed imaging, cell tagging, and single-cell analysis. On the other hand, the microarray technique has considerable prospects for high throughput profiling of intact tissue samples, however, one of the major challenges associated with microarray approaches is that oligonucleotide sequences can hybridize non-specifically with non-intended sequences. This activity is especially observed for cDNAs or ESTs that possess long features and for those genes with lengthy sequences thus displaying a greater degree of cross-hybridization, thus, cross-hybridization signal decreases significantly as the sequence similarities are reduced [166]. The oligonucleotide sequences that exhibit 80% sequence similarity show a decrease of ~5 to 10% of the perfect match whereas the effect of cross-hybridization is not serious for sequences that show fewer similarities. The specificity of oligonucleotide microarrays is greater than the cDNA microarrays but still, cross-hybridization is a major concern [167]. The degree of cross-hybridization in oligo arrays could be reduced by removing the sequences that have a high potential to cross hybridize. Optimization of hybridization conditions could be another strategy to reduce cross-hybridization. A major problem with smFISH is that the signal-to-noise ratio is quite low during the fluorescence images recording process and thus, this technique needs the use of such objectives provided with larger apertures (high numerical values) and higher magnification powers (100×). The challenge of low signal-to-noise ratio was resolved by multiplexed SeqFISH which used an additional hybridization chain reaction that increased the signal to noise ratio [138]. Additional use of rounds of hybridization increased the signal-to-noise ratio by application of noncombinatorial labeling and sequential smFISH hybridization technique [168].

LCM-RNAseq applied on FFPE tissue samples results in the extraction of low quantity and quality of mRNA and later analysis with LCM methods effects on the integrity of RNA. It is, therefore, imperative to improve LCM-RNAseq analysis techniques to increase the quality and stability of RNA biomolecules. This may include the use of an LCM instrument with a suitable IR laser and the use of an appropriate RNAseq library construction kit to avoid RNA damage [169]. The application of SMARTer Stranded Total RNASeq Kit v2 that can extract from 250 pg-10 ng RNA is one option. There are two other challenges of applying the LCM-RNASeq for spatial mapping of biomolecules especially RNA within tissue sections. First, the approach is time-consuming and can be used to analyze RNA from 10 to 100 cells whereas Bulk RNASeq can sequence RNA from $>1 \times 10^6$ cells. Secondly, the yield produced by LCM-RNASeq is of low quality as most RNA is degraded during LCM-RNASeq, therefore, it needs additional rounds of PCR cycles for amplification producing high PCR duplicates and biased gene expression profiles. These issues could be resolved by the application of alternative technologies or improved technological platforms. For instance, an approach developed by He et al. [170] involved the use of a new algorithm (ADVOCATE) with LCM-RNASeq derived data that could predict the expression profiles of different genes in the tissue sections of pancreatic ductal adenocarcinoma (PDA).

The application of DESI-MSI is largely hampered by spraying of the organic solvents and applying high-pressure nebulizing gases as well as the use of high voltages for evaluation of molecular entities in fresh tissue sections obtained from *in vivo* experiments. Thus, there is a need for the development of mass spectrometry techniques that could rely on direct analysis of cancer tissues. For instance, rapid evaporative ionization mass spectrometry or the iKnife technologies have been developed for *in situ* analysis of tumor tissues by combining mass spectrometry with an electrocauterization device [171]. Furthermore, the coupling of ultraviolet and infrared lasers with mass spectrometry has improved the resolution power of this technology [172].

One of the main challenges of spatial mapping by transcriptome analysis is that this technology cannot analyze the mRNA expression up to the single-cell level due to limitations created by microarray spot size and spacing. The advances made by Stahl's group in 2019 can visualize gene expression with an improved spatial resolution by 1400× and can detect spatial gene expression patterns at the level of a single-cell thus providing the prospect for the detection of cancer at early stages [142]. This will largely help to identify resistant tumor clones for targeted chemotherapies and will help to develop such platforms for improved immunotherapy responses. The detection of RNAs and proteins by hybridization-based approaches rely on the probes such as DNA and antibodies for digital spatial profiling while fluorescent *in situ* hybridization can detect only RNAs as the detection of proteins usually do not need the binding of multiple probes and mass spectrometry methods are more developed for proteins only.

Prospects should also include the need to spatially map the epigenome, both methylation and histone modification in the nucleus. The epigenetic state of the nucleic acid in the cells is critical to cancer progression and hence mapping of the methylation of specific molecules across the tissue can provide insight into the progression of cancer within a tissue. The development of molecular imaging techniques at the single-cell level has been helpful for the visualization of intercellular differences down to the epigenome level and in improving the understanding of tumor heterogeneity as compared with the bulk analysis techniques [173]. Although, mapping of DNA methylation in single cells has been reported based on genome-wide analysis using single-cell bisulfite sequencing (scBS-seq) [174], significant progress needs to be achieved towards the spatial mapping of methylation and histone modification in complex cancer tissue sections to understand the mechanism of disease progression.

6. Conclusions

Spatial information of biological entities with the advanced OMICS technologies can be advantageous in the prediction of biomolecules in tissue samples as these analytes are present in a pathological cancer tissues With the aid of OMICS techniques and computational approaches, spatial profiling of cancer tissues is progressing slowly as the techniques developed so far are at the early phase of development and further improvements are needed to increase the detection limit, number of different molecules detected from the same assay, and time required for each assay. Spatial mapping of analytes depends upon the integration of multiple types of information obtained from several OMICS techniques such as microarray, mass spectrometry, immunochemistry, single-cell RNA sequencing, fourth-generation RNA sequencing, laser cell microdissection, and digital pathology. These technologies have their inherent advantages and disadvantages, however, one of the unique features of these methods is that they can generate a spatial profile from a few molecules of RNAs or proteins (*i.e.*, IHC and immunofluorescence) up to >1000 RNAs and/or protein molecules with advanced OMICS techniques at a time in their positional context. Recent progress in DSP and machine learning techniques have opened a new window for multiplexed real-time spatial profiling of cancer tissues with great accuracy and remarkable resolution power, however, further improvement is still needed to resolve the long-standing issues of limited RNA profiling, unable to sequence the targeted genes and visualize unknown genes in their physical locations.

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Data availability statement

This is a review article and does not contain any raw data.

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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