



Spatiotemporal dissection of tumor microenvironment via in situ sensing and monitoring in tumor-on-a-chip

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ABSTRACT

Real-time monitoring in the tumor microenvironment provides critical insights of cancer progression and mechanistic understanding of responses to cancer treatments. However, clinical challenges and significant questions remain regarding assessment of limited clinical tissue samples, establishment of validated, controllable pre-clinical cancer models, monitoring of static versus dynamic markers, and the translation of insights gained from *in vitro* tumor microenvironments to systematic investigation and understanding in clinical practice. State-of-art tumor-on-a-chip strategies will be reviewed herein, and emerging real-time sensing and monitoring platforms for on-chip analysis of tumor microenvironment will also be examined. The integration of the sensors with tumor-on-a-chip platforms to provide spatiotemporal information of the tumor microenvironment and the associated challenges will be further evaluated. Though optimal integrated systems for in situ monitoring are still in evolution, great promises lie ahead that will open new paradigm for rapid, comprehensive analysis of cancer development and assist clinicians with powerful tools to guide the diagnosis, prognosis and treatment course in cancer.

1. Introduction

During the initiation and progression of cancer, tumor cells trigger neighboring cellular, molecular, and physical changes and interact with ambient environment (Anderson and Simon, 2020). The cellular and extracellular components surrounding tumor cells together form the so-called tumor microenvironment (TME), a complex and continuously evolving entity in promoting cancer progression (Truffi et al., 2020). The cellular components in TME consist of tumor cells and tumor stromal cells, including fibroblasts, endothelial cells, pericytes and immune cells. The non-cellular components are mainly extracellular matrix (ECM), extracellular vesicles, cell-released cytokines, and metabolic factors (Baghban et al., 2020; Labani-Motlagh et al., 2020; Milotti et al., 2020; Ribeiro Franco et al., 2020)(Fig. 1). The mutual interactions between tumor cells and cellular and non-cellular components in TME compromise the tissue integrity, and facilitate carcinogenesis and tumor progression (Jahanban-Esfahlan et al., 2019; Seidi et al., 2018). Dynamic exchanges among tumor cells, reactive non-neoplastic cells, and

ECM in TME play essential roles in tumorigenesis by regulating clonal evolution, cancer heterogeneity, epithelial-mesenchymal transition (EMT), migration, invasion, angiogenesis, apoptosis and drug resistance (Baghban et al., 2020; Cova et al., 2019; Li et al., 2018; Tsao et al., 2016; Ungefroren et al., 2011). Oncology studies have suggested that a comprehensive analysis of the interplays among the complex components in TME is essential to unveil the hidden mechanisms of tumor growth and metastasis and to the development of novel therapeutics to improve patient outcome (Jahanban-Esfahlan et al., 2018). The emerging cancer immunotherapies, featured as the future of cancer treatment, have showcased the latest development in deciphering the tumor immune microenvironment. However, the heterogeneous patient responses are still not well clarified (Waldman et al., 2020), highlighting the importance of systematic investigation of TME to gain mechanistic insights in tumor-immune interactions.

Various cancer model systems have been developed for cancer research, anti-cancer drug screening, and therapeutic discoveries, such as *in vitro* two-dimensional (2D) or 3D cell culture and animal models

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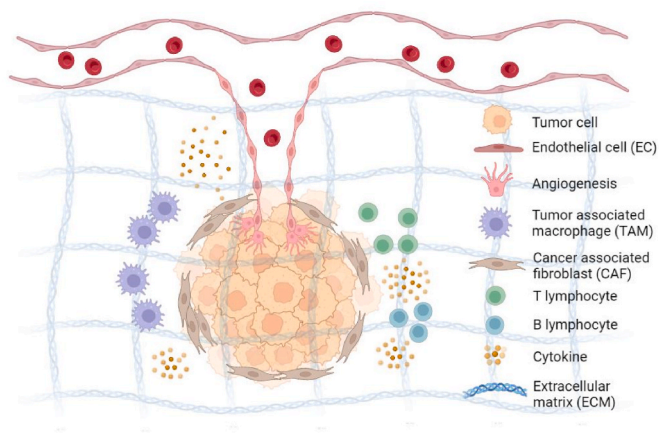


Fig. 1. Components of the tumor microenvironment. Tumor microenvironment consists of multiple cells including tumor cells, endothelial cells forming vasculature participating in angiogenesis and metastasis, cancer associated fibroblasts, tumor associated macrophages, and lymphocytes. Cytokines and chemokines released by malignant and stromal cells inhibit the function of resident immune cells, leading to the TME immunosuppression. Extracellular matrices facilitate invasion of tumor cells through matrix remodeling. Metabolic factors like pH and glucose are suppressed in tumor milieu. Lastly, hypoxia, the lack of oxygen, also plays a key role in the evolution of tumors, contributing to tumor invasion and metastasis.

using human cell lines or patient-derived xenografts (PDXs) (Ribeiro Franco et al., 2020). The immortalized cancer cell lines, characterized by low cost, high reproducibility, and ease of use, have been widely employed for drug screening and *in vitro* cell biology studies (Efremov et al., 2013; Ehsan et al., 2014; Fong Eliza Li et al., 2013; Sokol et al., 2016). However, the cell cultures are not ideal to study TME due to the lack of spatial architecture and arrangements, and limited capability to recapitulate the system nature of tumor (Tsai et al., 2017). The PDXs retain a more comprehensive tumor molecular profile and can produce complex cancer-stroma interactions *in vivo*, but the time-consuming and labor intensive generation processes and the incompatibility with human immune system have become the major obstacles for their clinical applications in cancer immunotherapies (Begley and Ellis, 2012).

Recently, organ-on-a-chip (OOC), by growing microtissues inside a microfluidic chip, provides a unique opportunity to *in vitro* recapitulate the *in vivo* structures and physiological conditions of human organs, rendering them an ideal platform to offer complex model systems with better engineered microenvironments in cancer research and treatment (Leung et al., 2022; Liu et al., 2021). OOCs are mostly fabricated by soft lithography based on polydimethylsiloxane (PDMS), an elastomer with optical transparency facilitating imaging, and high biocompatibility and fabricability (Qin et al., 2010; Wang et al., 2012). During the soft lithography process, the silicon wafer is firstly coated with photoresist, then exposed to UV light under a mask and etched to generate the mold of the OOCs. PDMS will be cast and solidified on the mold, and peeled off with designed microfluidic patterns by the so-called replica molding process. Besides, inject molding is suitable for thermoplastic-based (e.g., polycarbonate (PC) and polymethylmethacrylate (PMMA)) device fabrication, excelling in mass production efficiency and meeting the need of high throughput testing (Chen et al., 2010; Galateanu et al., 2022b; Ren et al., 2013). 3D printing in OOC fabrication emerges in recent years due to the features of assembly-free and rapid prototyping of complex structures with high freedom of design (Prabhakar et al., 2021). Such layer-by-layer method ensures a high spatial resolution, controllability and repeatability in OOC establishment (Bishop et al., 2017).

Leveraging the advances of OOC technique, TOC systems have emerged as a new tool for creating functional 3D *in vitro* human tumor models for oncology research (Caballero et al., 2017; Shang et al., 2019;

Sun et al., 2019; Tsai et al., 2017). By reconstructing TME on chip with key cancer-associated cellular and non-cellular components, TOC allows *in vitro* studies of simulated *in vivo* tumor pathological processes like expansion, angiogenesis, metastasis, and tumor-stroma-immune cell interactions in the TME (Albanese et al., 2013; Wang et al., 2018). In particular, the reproduced specific traits of TME in TOC, such as the biological gradient, niche factors, dynamic cellular interactions, and 3D configurations of the tumor and stroma cells, have become critical factors to generate reliable, well-controlled preclinical models for studying tumor evolution, cancer-immune interactions, and developing novel chemo- and immune-therapies, of which conventional cell cultures or animal models are not amenable to do (Astolfi et al., 2016; Fetah et al., 2019; Han et al., 2016; Kennedy et al., 2019; Kumar and Varghese, 2019; Shirure et al., 2018).

Despite the continuous development in TOC for cancer research, accurate and real-time measurements of tumor-related pathological processes in a biomimetic TME remain challenging. Particularly, quantitative and spatiotemporal analysis of various cellular and molecular markers in the TME is essential for in-depth characterization of the TME, determination of new biological mechanisms, and validation of clinical interventions. To obtain such readouts containing cellular and molecular insights of TME in TOC, various sensing and analytical technologies, such as immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR), have been exploited for on-chip and off-chip analysis. Nonetheless, most of the current methods require complicated and time-consuming experimental procedures and bulky instrumentations, thus fall short to provide *in situ*, real-time sensing and monitoring of the rapid changing characteristics in the TME.

In this review, we discuss the status of TOC research, highlight the emerging needs for *in situ* monitoring in TOC, and evaluate the advanced imaging, sensing and analytical tools that could be integrated with TOCs to acquire spatiotemporal information to tightly monitor and regulate the TME. We further discuss the critical technical challenges and future direction in the development of integrated TOC technology to deepen the understanding of TME and facilitate its translational applications in cancer prevention and treatments.

2. Critical features of TME

The main cellular components in TME include cancer associated fibroblasts (CAFs) that stimulate cancer cell invasion and proliferation (Ribeiro Franco et al., 2020), endothelial cells (ECs) that form vasculatures to promote tumor growth and metastasis (Chen et al., 2013), and immune cells like tumor associated macrophages (TAMs) that assist tumor cells to escape from immune surveillance (Zhou et al., 2020). Detailed characterization of these cellular features in TME can lead to a better understanding of the pathophysiological interactions in TME, which is key to the development of novel strategies for cancer treatments (Ahn et al., 2017). For example, measuring the proliferation and viability of different types of cells can reveal the stimulation or suppression factors in TME and further evaluate their synergetic or antagonistic effects and immunocompetence. Investigations on the migration of cells offer cues of tropism and chemotaxis in the mutual interactions between tumor and neighboring cells. Besides, angiogenesis, the formation of new blood vessels from existing vasculature, is regarded as a main facilitator of nutrient supply in tumor and a major route for cancer cells to enter the circulation system (Ahn et al., 2017; Papetti and Herman, 2002). Thus, the study of vascular perfusion in TME can provide valuable insights on the role of vasculature changes in tumor growth and metastasis.

Non-cellular components in the TME, including cytokines, growth factors, ligands, small RNAs, DNA, soluble factors, metabolite, and ECM, have been evidenced to direct the interactions with cellular components and play a critical role in shaping the tumor niche and promoting the tumor progression (Patel et al., 2018). For instance, the TME cytokines, especially the anti-inflammatory ones (Baldridge et al., 2011; Goldstein

and Kim, 1993; Grivennikov and Karin, 2011), create a tumor-supportive microenvironment via inhibition of anti-tumor immunity and exerting direct tumor-promoting functions (Kartikasari et al., 2021). Thus, the analysis of cytokine components in TME can provide a better understanding of the complex, mutual, and dynamic signal network in malignant transformation (Elsawa et al., 2011). Metabolic factors in TME (i.e., nutrient deficiency, hypoxia, acidity) are another type of major contributors to the modulation of immune metabolism and reprogramming of immune cells towards pro- or anti-inflammatory phenotypes (El Sayed et al., 2021). In addition, the ECM surrounding the cancer cells serves as a structural and biochemical support and regulates cell-cell or cell-matrix cross-talks (Brassart-Pasco et al., 2020). In TME, ECM differs significantly from that in normal niches, which is strongly associated with intra-tumoral signaling, transport mechanisms, metabolisms, oxygenation, and immunogenicity. With such effects, ECM exerts great influences on the malignancy and growth of tumor as well as its response to treatments (Henke et al., 2020). The following sections present a brief introduction of current methods to characterize these cellular and non-cellular components of TME on TOC.

3. Modeling TME with TOC

Generally, TOC allows for the recreation of various cellular and non-cellular TME features such as multicellular interactions, ECM-based biochemical properties, biophysical cues like hypoxia and their gradients (Ando et al., 2017; Apoorva et al., 2018). Tumors, together with vasculatures, and stromal and immune cells, could grow on a chip with a complex tissue structure either self-organized or spatially organized by design, mimicking the *in vivo* counterparts (Fig. 2A), outperforming 2D models and 3D tumor spheroids/organoids models in complexity, physiological relevance, repeatability or controllability (Choi et al., 2015; Jeong et al., 2016; Tsai et al., 2017).

Vasculature, a key feature in TME affecting tumor progression and metastasis but mostly lacking in 2D culture and 3D spheroids/organoids,

can be well maintained in TOC to achieve a high degree of biomimicry to human body. Specifically, a vascularized TOC can recapitulate the perfusable tumor vascular network, mimicking cellular and biochemical transport and drug delivery (Hu et al., 2022; Jeon Jessie et al., 2015; Kameda et al., 2022; Kim et al., 2013; Nashimoto et al. 2017, 2020; Zhang et al., 2021). Although endothelial cells have been mixed in 3D tumor spheroids, such co-culture strategy cannot faithfully model the vascular network *in vitro* because the grown structures are not perfusable (Ehsan et al., 2014; Shoval et al., 2017). By loading the tumor spheroid in the center of the microfluidic device and vascularizing it with angiogenic sprouts from neighboring microfluidic channels, a TOC platform established a perfusable vascular network enabling the transport of nutrition, oxygen and drugs and evaluation of tumor activities with intraluminal flow (Fig. 2B)(Nashimoto et al., 2020). Drug administration under perfusion condition was conducted and showed findings cannot be reflected by traditional static culturing condition, making the TOC a promising platform for drug screening.

Apart from vascular networks, complex interactions of stromal and immune cells with tumor cells in TME can be studied using the biomimicking co-culture system of TOC as well (Aref et al., 2018; Bersini et al., 2014; Businaro et al., 2013; Chen Michelle et al., 2018; Cui et al. 2018, 2020; Hsu et al., 2012; Jeon Jessie et al., 2015; Ma et al., 2020; Mattei et al., 2014; Menon et al., 2014; Moore et al., 2018; Nguyen et al., 2018; Sung et al., 2011; Zheng et al., 2016). Conventional 2D and 3D models are oversimplified thus cannot reconstitute the *in vivo* architecture and corresponding niche containing stromal and immune cells. Ma et al. designed a TOC mimicking bone marrow structure with central sinus, medullary cavity and endosteal space (Fig. 2C)(Ma et al., 2020). By introducing essential niche cells of bone marrow like bone marrow stem cells, osteoblasts, and bone marrow mononuclear cells on a microfluidic chip, they integrated key features replicating *in vivo* BM tissue and compared the TOC TME with the *in vivo* counterparts. Besides, the proposed TOC enabled dynamically monitoring of leukemic cells and BM niche interactions and TME evolution, which cannot be achieved by animal models. By applying niche-cotargeting treatments on chip, the

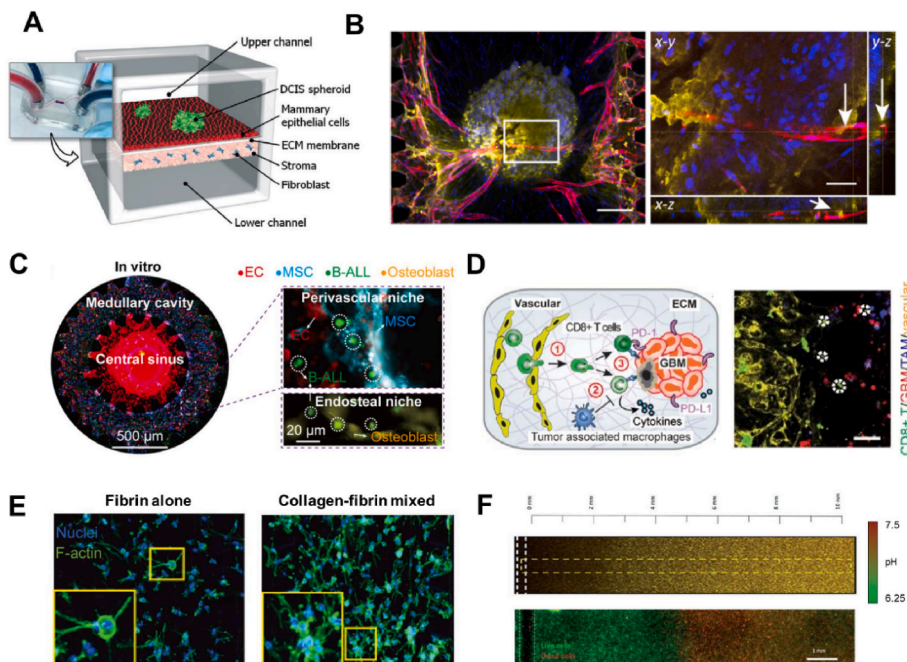


Fig. 2. Modeling TME on tumor-on-a-chip with multiple components. (A) A human breast-cancer-on-a-chip replicating the microarchitecture of breast ductal carcinoma with integration of tumor spheroids, mammary ductal epithelial cells, and mammary fibroblasts. Adapted with permission. Copyright 2015, the Royal Society of Chemistry. (Choi et al., 2015). (B) Immunofluorescence images of the tumor spheroid integrated with the blood vessels on a vascularized TOC. Lumen structure of vasculatures was observed, indicating the perfusability of vascular network. Adapted with permission. Copyright 2020, Elsevier. (Nashimoto et al., 2020). (C) Whole scan of the leukemic BM niche system. Compartments on chip resembled the *in vivo* counterparts like central sinus and medullary cavity with niche cells. Adapted under the terms of the CC-BY-NC license. Copyright 2020, the Authors (Ma et al., 2020). (D) Immunosuppressive TME on GBM-on-a-chip. The reconstituted GBM TME recapitulated the infiltration of T cells and interactions among T cells, TAMs, and GBM tumor cells. Adapted under the terms of the CC-BY license. Copyright 2020, the Authors (Cui et al., 2020). (E) Various components of ECM loaded on TOC revealed the distinct phenotypic changes of cancer cells in response to different ECM conditions. Adapted with permission. Copyright 2017, Wiley. (Chung et al., 2017). (F) pH gradient and necrotic region were formed on TOC under metabolic starvation gradients. Adapted with permission. Copyright 2019, the Royal Society of Chemistry. (Ayuso et al.,

TOC showed great potential to be translated as a preclinical model for patient-specific therapy-screening. In a separate study, Cui et al. recapitulated the immunosuppressive TAMs, CD8⁺ T cells, and brain tissue-mimicking hydrogel in a glioblastoma (GBM)-on-a-Chip TME model (Fig. 2D)(Cui et al., 2020). To bridge the gap between the pre-clinical findings in animal models/patient explant models and clinical results based on patients, the designed TOC created a humanized TME by adopting patient-derived samples of different GBM subtypes with higher biological relevance to human bodies. The chip achieved spatiotemporal resolution analysis of TME with recording the migration trajectories of infiltrated CD8⁺ T on chip. By investigating the heterogeneous responses to anti-PD-1 therapy across different GBM subtypes and screening novel combinational immunotherapy with co-targeting TAMs, the TOC demonstrated the ability in predicting immunotherapy efficacy and facilitating precision immune-oncology as a high-throughput low-cost method.

ECM, another essential component in TME, affects the growth and malignancy of tumor and its response to therapies (Henke et al., 2020). By including ECM components in TOC, a 3D structural framework for cell growth with mechanical context and dimensionality can be achieved *in vitro* (Anguiano et al., 2017; Carvalho et al., 2019; Chung et al., 2017; Del Piccolo et al., 2021; Huang et al., 2009; Kwak et al., 2014; Shin et al., 2014; Sung et al., 2011; Young, 2013). In a microfluidic platform, by co-culturing cancer cells and fibroblasts in components-varied ECM, the growth and invasion of tumor cells of different types were characterized (Fig. 2E)(Chung et al., 2017). Due to the structural complexity and modular design of the TOC, the platform showed higher controllability and repeatability and enabled a more comprehensive study of the crosstalk between different kinds of ECM and tumor cells than 2D, 3D or animal models. It was found that, compared to fibrin alone, ovarian cancer cells exhibited larger cytoplasmic volume and more clustered nuclei when cultured in collagen-fibrin mixed ECM, highlighting the role of ECM composition, concentration, and stiffness in tumor cells proliferation and revealing the advantages of TOC in combining different TME components to study their interactions.

The heterogeneity of metabolic factors like oxygen, nutrient, pH, growth factors and cell viability gradients altered the progression of tumor and drug resistance, especially in solid tumors (Bader et al., 2020; Shi et al., 2020). Due to the high amenability and flexibility, TOCs have been designed to generate these TME cues, mimicking the *in vivo* tumor metabolism (Albanese et al., 2013; Ayuso et al. 2019, 2020; Bhattacharya et al., 2020; DelNero et al., 2015; Menon et al., 2014; Palacio-Castañeda et al., 2020; Song et al., 2018; Welton et al., 2014). Current widely applied 3D spheroids to reconstitute the metabolic milieu *in vitro* are oversized for imaging and not ideal to study factors of interest individually. By loading tumor cells in the center chamber and using a lumen on the flank of the chamber as blood vessels for medium perfusion, a microfluidic tumor slice model created the gradients of viability, proliferation, and pH in three different cell populations (proliferating, stationary, and dead cells) along the chip (Fig. 2F)(Ayuso et al., 2019). The established controllable TME can be characterized by on chip imaging, decoupled from other unconcerned factors, and disassembled to retrieve cells for downstream analysis, which cannot be afforded by multicellular tumor spheroid models.

4. Current methods to analyze TME on TOC

Based on the unique features presented by the TME and the various TOC models been developed, we summarized the current methods that have been applied for TME analysis in three main categories: fluorescence-based methods for visualizing cellular components and ECM in TME, ELISA-based assays for molecular analysis, and gene expression analysis with RT-PCR and single-cell RNA sequencing (scRNA-seq) techniques.

4.1. Fluorescence-based methods

Fluorescence labelling has been the most used approach for identification of specific biomolecule markers and visualization of molecular and cellular features and biological processes in TME. For example, in an *in-vitro* cancer model for simulating the recruitment of pericytes to angiogenic sprouts, cells were stained and the fluorescent signals showed the attachment of pericytes to the endothelial-derived collagen IV with stretched morphology (Fig. 3A)(Kim et al., 2013). Cell-labeling reagents turned highly fluorescent in a reducing environment for viable cells, allowing qualitative analysis of cell proliferation in the TME (Menon et al., 2014). The quantification of cells can also be achieved by double-stranded DNA (dsDNA) fluorescent stain (Lai et al., 2020). Cell staining to differentiate live and dead cells has been widely used to characterize the cell viability on-chip, exemplified by the quantification of anti-PD-1 antibody treatment effect to determine the CD8 T-cell-mediated tumor cell cytotoxicity (Fig. 3B)(Aref et al., 2018), and the induced apoptosis of target GBM cells treated with combined immunotherapies (Fig. 3C)(Cui et al., 2020). Fluorescent proteins, due to their excellent biocompatibility, have been utilized to monitor cell migration by transfection of target cells without compromising the cell viability and function. Breast cancer cells labeled with green fluorescent protein (GFP) were cultured on a microfluidic device to visualize the cell migration during tumor growth (Fig. 3D)(Mi et al., 2016). By tagging cocultured tumor cells and endothelial cells with different fluorescent dyes, the trans-endothelial invasion of tumor under the stimulation of chemokine in TME was observed *in vitro* (Fig. 3E)(Zhang et al., 2012). In another microfluidic tumor-vascular model, fluorescent dextran was applied to measure the diffusive permeability of the vascular networks to study the cancer cell invasion and intravasation. The vascular networks were observed to be more permeable than normal vessel in the presence of tumor cells (Fig. 3F)(Nagaraju et al., 2018).

Apart from cellular components, ECM like laminin, fibronectin, collagen and hyaluronic acid can be fixed with immunofluorescent dyes to provide molecular insights about the compositions (Cui et al., 2020). To measure the robustness and stiffness of ECM under shear stress, fluorescent beads have been applied in a tri-layer hydrogel system to determine whether the interlayer structures were differentially compressed at varied flow rates (Aung et al., 2016). Besides, reactive oxygen species (ROS), an important metabolic factor in cell signaling and homeostasis, was measured by an oxidative stress indicator in a microfluidic co-culture system to monitor the tumor-stromal interactions (Menon et al., 2014). The labeling reagent diffused into the cells and the oxidation resulted in a fluorescent adduct trapped inside the cell.

4.2. ELISA-based methods

In TOC studies, the “gold standard” method to quantify the secretory proteins (i.e., cytokines) is ELISA: the detected ligand is against the specific antibody linked to an enzyme which produces signals in substrate-enzyme reactions (Fig. 4A)(Zhang et al., 2014). Such assay can be transformed to other sandwich-based sensing platforms based on the signal transduction mechanisms, including color change, bioluminescence, chemiluminescence, electrochemiluminescence, etc. Advances in membrane-, microbeads-, and microarray-based technologies have created new tools that further allow high-throughput, multiplex detection in these assays. For instance, ELISA was applied to determine the amount of TGF- β 1 in the conditioned media of a lung cancer-on-a-chip, and to confirm its functional roles in the interactions between cancer cells and fibroblasts (Hsu et al., 2011). Massive parallel analysis of ten angiogenesis-related cytokines were achieved by a microarray based immunoassay for identification of signaling molecules in tumor-vascular microenvironment (Fig. 4B)(Nagaraju et al., 2018). Bioluminescence complementation provides a facile approach to validate and quantify the protein-protein interactions (Luker and Luker, 2011). To investigate chemokine (different isoforms of CXCL12) guided migration of cancer

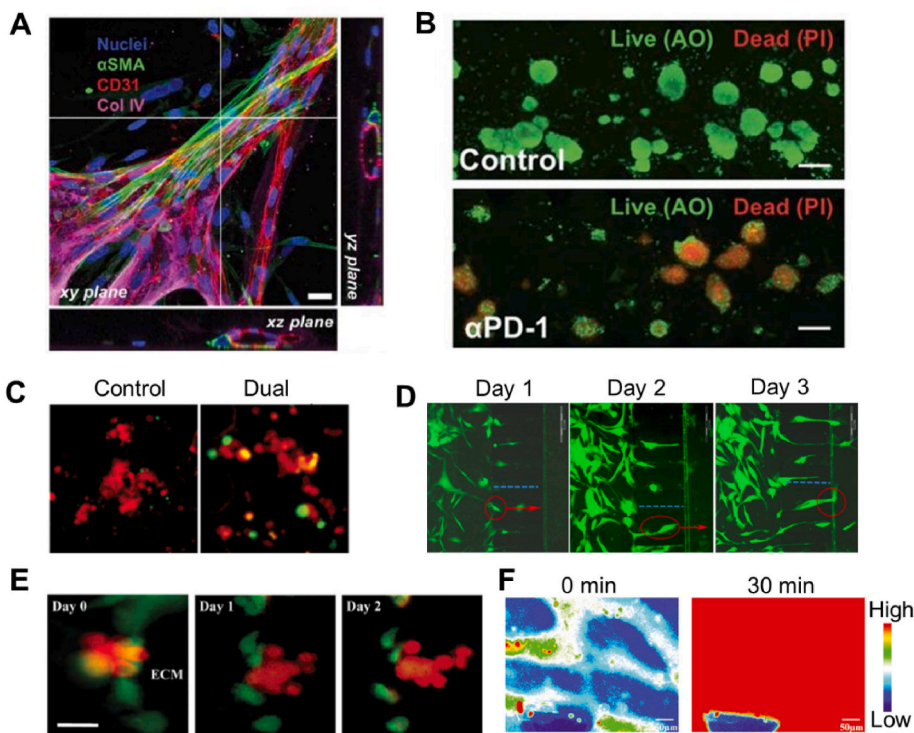


Fig. 3. Fluorescence-based methods. (A) Immunostaining image of a pericyte-decorated blood vessel: microvascular network (CD31, red) covered with pericytes (α-SMA, green) Adapted with permission. Copyright 2013, the Royal Society of Chemistry (Kim et al., 2013). (B) Live/Dead imaging of organotypic tumor spheroids with acridine orange (AO) and propidium iodide (PI) labelling. Adapted with permission. Copyright 2018, the Royal Society of Chemistry (Aref et al., 2018). (C) Increased apoptosis of tumor cells when receiving combinational immunotherapy was shown by caspase-3/7 activation. Adapted under the terms of the CC-BY license. Copyright 2020, the Authors. (Cui et al., 2020). (D) Temporal images of GFP-labeled cancer cell migration, where red circle indicated the same cancer cell in different time points. Adapted under the terms of the CC-BY license. Copyright 2016, the Authors (Mi et al., 2016). (E) Transendothelial invasion of tumor aggregates induced by CXCL12, where cancer cells were labeled with CellTracker Red (red) and endothelial cells were labeled with CellTracker Green (green). Adapted with permission. Copyright 2012, the Royal Society of Chemistry. (Zhang et al., 2012). (F) Heat map of fluorescence intensity denotes diffusion of FITC-dextran from a more permeable vascular network due to the presence of tumor cells. Adapted with permission. Copyright 2018, Wiley. (Nagaraju et al., 2018).

cells and the role of CXCR7 in the migration of CXCR4, CXCL12-isoforms to Gaussia luciferase and luciferase complementation between CXCR4 or CXCR7 and β -arrestin 2 were exploited (Cavnar et al., 2014). By replacing the enzyme tag with chemiluminescence or electrochemiluminescent labels, sandwich-based immunoassays were employed for on-chip membrane-based proteomic analysis and cytokine detection in TMEs (Fig. 4C and D) (Hassell et al., 2017; Lee et al., 2018a; Zhao et al., 2020a). Recently, microbeads-based sandwich immunoassays have emerged as a highly multiplex detection platform that can conduct up to 100 types of protein analysis simultaneously (Fig. 4E) (Khalifian et al., 2015). Based on this method, time-lapse cytokine profiling was performed in a 3D microfluidic platform modelling immune checkpoint treatment with only a small volume of conditioned media, demonstrating the evident evolution of cytokine and growth factor secretion under different treatments over time (Fig. 4F) (Aref et al., 2018).

4.3. Gene expression analysis

Gene expression analysis provides genetic details and transcriptomic signals of the functional RNA species or protein products as key molecular classifiers in TME. Currently, the most widely applied techniques are PCR, expression microarrays, and RNA-seq (Segundo-Val and Sanz-Lozano, 2016).

Real-time PCR (RT-PCR) has been broadly accepted as a general approach for gene quantitation as well as protein expression analysis due to its large dynamic range, tremendous sensitivity, high sequence-specificity, little post-amplification processing, and amenability to increasing sample throughput (Wong and Medrano, 2005). In a 3D microfluidic tumor vascular model, RT-PCR was applied on measuring the expression levels of target genes in cancer cells during co-culture or mono-culture to determine the effect of wall shear stress upon exposure to endothelial cells (Buchanan et al., 2014). With microarray assay, thousands of genes from a sample can be detected simultaneously and have been applied to study tumor cell hypoxia response for cancer classification (DelNero et al., 2015). Single cell mRNA sequencing (scRNA-seq) technology examines the genetic information of individual

cells with optimized next-generation sequencing (NGS) technologies, which can identify and characterize cellular compositions and provide a better understanding of individual cell function in the context of its microenvironment (Eberwine et al., 2014). A leukemia-on-a-chip study comparatively mapped the B-ALL subtype-specific niche signals utilizing scRNA-seq and validated that the niche-enhanced downstream signaling and cellular quiescence in B-ALL blasts could promote chemotherapy resistance (Ma et al., 2020).

4.4. Other analysis methods

Phase-contrast microscope can produce high contrast images of transparent biological samples, rendering it an ideal technique for on-chip cell observation. The angiogenic sprouting morphogenesis of endothelial cells towards leukemia cells were observed with phase-contrast microscope (Fig. 5A) (Zheng et al., 2016), in which the small differences in thickness and refractive index of the cells would induce phase changes of light and could be easily traced by the brightness change via transformation of phase condenser and phase retardation plate. Second-harmonic generation (SHG) microscopy is highly sensitive to the collagen fibril/fiber structure and diseases-related conditions like cancer, fibrosis and connective tissue disorders, and has thus emerged as a powerful tool for imaging fibrillar collagen in ECM (Chen et al., 2012). In a 3D multicellular co-culture microfluidic platform, two different adjoined collagen gels were observed with SHG, revealing that an acellular gel remained intact while a gel channel containing cancer cells displayed empty areas devoid of collagen fibrils, demonstrating that cancer cells can remodel ECM by proteolysis and tractional stress (Fig. 5B) (Huang et al., 2009). Atomic force microscope (AFM) was implemented to measure the tissue elasticity on chip under conditions of tumor organoids culturing with or without fibroblasts (Lai et al., 2020). Each AFM indentation measurement was performed using a custom spherio-conical tip with a polystyrene bead to cantilevers, indicating organoids-fibroblasts crosstalk increases tissue elasticity. To detect metabolic factors, a microfluidics based-multiparametric microphysiometry system was developed by integrating oxygen or pH sensors upstream in the inlet channel, inside the cell cultivation area, and

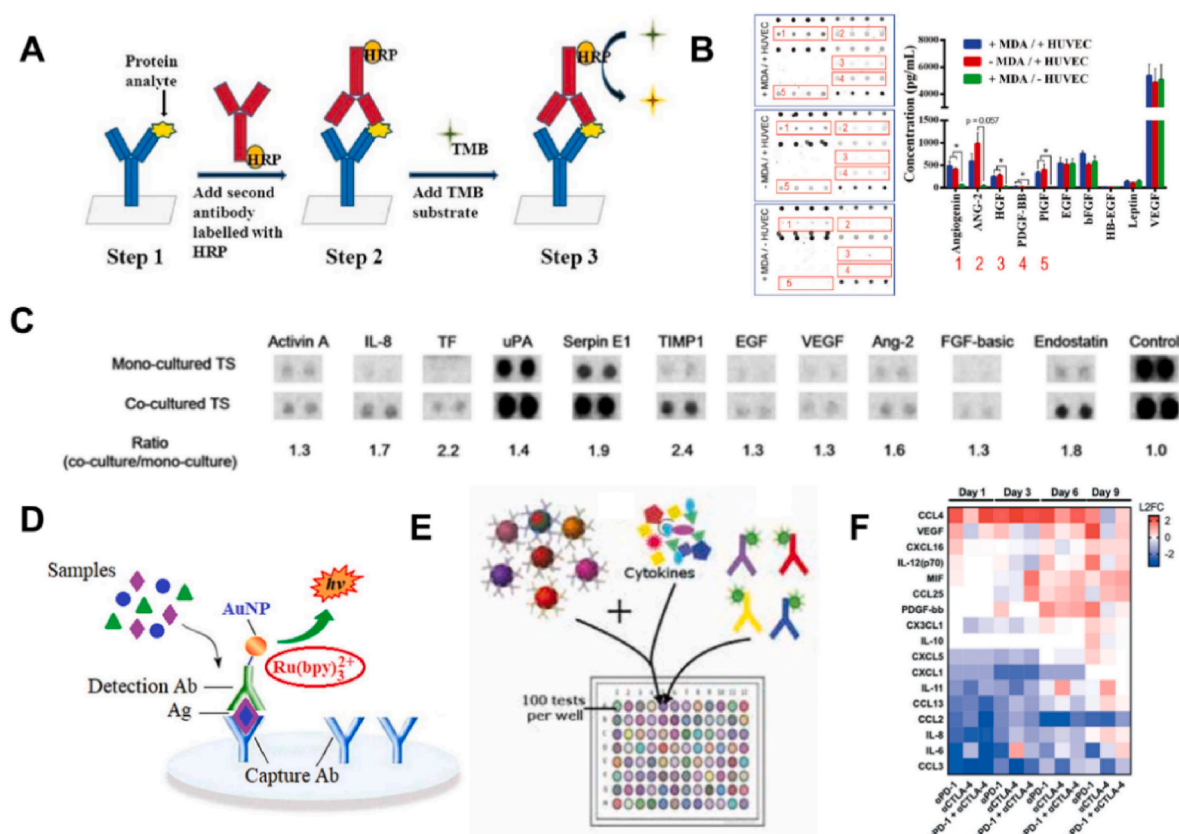


Fig. 4. ELISA-based analysis. (A) Concepts of ELISA. Adapted with permission. Copyright 2014, the Royal Society of Chemistry (Zhang et al., 2014). (B) Profiling ten angiogenesis-related cytokines in the tumor-vascular TME on chip with multiplex ELISA. Adapted with permission. Copyright 2018, Wiley (Nagaraju et al., 2018). (C) Chemiluminescence-based proteome array analysis of angiogenesis and metastasis-related cytokines in tumor spheroids-stellate cells co-culture system. 11 factors among 55 tested cytokines showed significant statistic differences. Adapted under the terms of the CC-BY license. Copyright 2018, the Authors (Lee et al., 2018a). (D) Concepts of a sandwich-based ECLIA using gold nanoparticles as both label and co-reactant. Adapted with permission. Copyright 2020, Elsevier (Zhao et al., 2020a). (E) Principle of microbead-based sandwich immunoassay. Plastic beads with red and infrared fluorescent dyes provide unique signatures to samples while the detection antibodies conjugated with a reporter dye are added to measure cytokines. Adapted with permission. Copyright 2015, Elsevier. (Khalifian et al., 2015). (F) Time-lapse heatmap of secretory cytokines under different immune checkpoint blockade treatments from tumor spheroids on a chip measured by bead-based immunoassay. Adapted with permission. Copyright 2018, the Royal Society of Chemistry. (Aref et al., 2018).

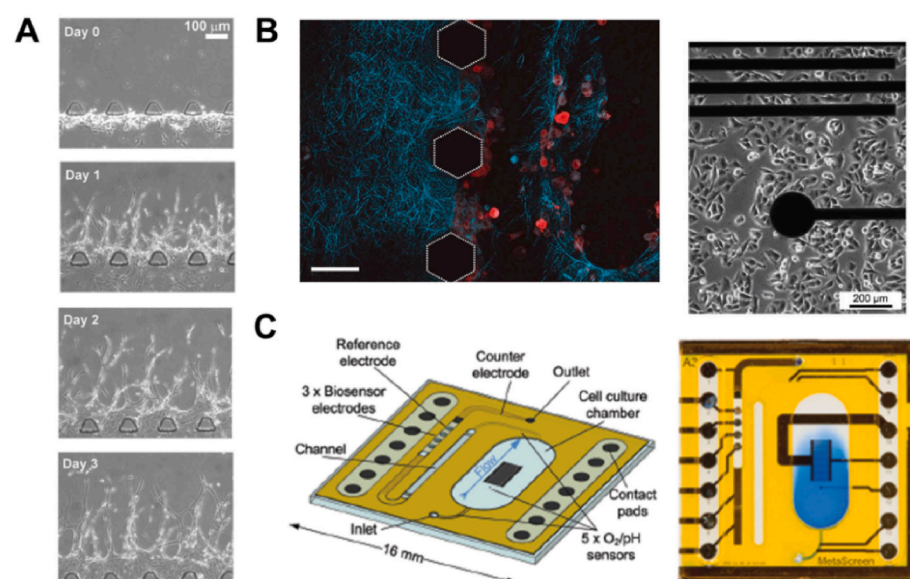


Fig. 5. Other current analysis methods. (A) Phase contrast microphotographs of migration of endothelial cells towards leukemia cells over time on a bio-mimetic angiogenesis chip. Adapted with permission. Copyright 2017, Wiley (Zheng et al., 2016). (B) The degradation of collagen fibrils (in blue) caused by the migration of breast cancer cells (in red) was imaged via SHG within a microfluidic device. Adapted with permission. Copyright 2009, the Royal Society of Chemistry. (Huang et al., 2009). (C) Metabolic factors sensing chip by integrating O₂, pH, glucose and lactate biosensors in one microfluidic device. Adapted with permission. Copyright 2014, the Royal Society of Chemistry (Weltin et al., 2014).

downstream in the outlet channel. To measure nutrition factor like glucose or lactate, the analytes reacted with O_2 and the produced electrons were detected on-chip based on amperometric measurements (Fig. 5C) (Weltin et al., 2014).

4.5. Limitations of current measuring methods

Based on the above discussions, we summarized current measuring technologies that have been utilized in investigating TME on TOC and categorized them based on different key components (stromal cells, vasculatures, immune cells, non-cellular components) studied in TME (Table 1).

Although various methods have been successfully used to characterize the cellular and non-cellular components of TME in TOC models, assessments of cell response by these approaches are mostly labor- and time-consuming and only provide end-point results, which pose significant challenges towards real-time and in situ analysis of TME on chip. Taking immunofluorescence as an example, it requires invasive collection of cells, *ex situ* cell fixation, and overnight incubation, and suffers from photobleaching for real-time imaging and time-lapse monitoring. For secretory protein analysis, current ELISA-based methods are mostly performed off-chip and fall short to provide sensitive detection especially for rare biomarkers or at low cell count. Moreover, the collection of the target containing medium from TOC would unavoidably disrupt the TME and result in an averaged measurement that cannot pinpoint the original state of the system (Kumar and Varghese, 2019). Most importantly, the intratumor heterogeneity and the rapidly evolving status of TME, which foster tumor evolution and adaption, have become the major obstacles for continuous assessment of TME. These emerging challenges have spurred the demand for new technologies that can be well adopted to dissect the spatial and temporal information of TME in the TOC.

5. Technologies available for in situ, real-time sensing, monitoring, and regulation of TME

To overcome the obstacles mentioned above, novel sensing technologies are critically needed with the capability to allow in situ, real-time sensing, monitoring, and regulation of TME and should be highly compatible with a TOC. Comparing to fluorescence-based, ELISA-based, or traditional gene analysis methods, novel fluorescence molecular imaging, real-time detection assays, label free biosensors, and on-chip high-throughput RNA/DNA sequencing offer diverse features in analyzing a variety of important targets in TME (cytokines, growth factors, ligands, RNA, DNA, metabolite, ECM, etc.) and provide promising sensing performance, particularly in spatiotemporal measurements.

5.1. Real-time detection biosensors/assays for extracellular protein analysis

Secretory proteins in TME, such as cytokines, play a significant role in the cellular processes that drive the malignant progression. Therefore, a comprehensive analysis of secretory proteins in TME is pivotal for us to understand the complex malignant progression, and mutual interaction between TME and malignant transformation. Current methods for secretory protein analysis are used tangentially to access the TME that only present a global snapshot of the protein profiles at the time of analysis. To achieve the in situ real-time detection of secreted proteins in TME, we summarized state-of-art biosensors/assays that can be potentially integrated with TOCs and classified them based on sensing modalities.

5.1.1. Electrochemical biosensors

Electrochemical detection features low cost, fast response, simplicity and high sensitivity, and is ideally suited for remote and/or embedded

real-time detection of biomarkers in TME on TOCs (Ronkainen et al., 2010). Electrochemical biosensors have been applied in TME of TOCs, such as the detection of cellular metabolites (i.e., lactate and glucose) (Misun et al., 2016), and secreted proteins (i.e., albumin and glutathione-S-transferase- α (GST- α) (Shin et al., 2017)). The signal transduction is based on label-free electrochemical impedance, charge transfer induced by electrochemically activated labelling molecules, or oxidation reaction catalyzed by enzymes. For example, Ali's group reported aptamer and antibody based electrochemical immunobiosensors for continual embedded monitoring of cell secreted biomarkers (Riahi et al., 2016; Zhang et al., 2017). A label-free electrochemical immunobiosensor was developed for automated on-chip monitoring of liver function biomarkers GST- α and albumin, and cardiac biomarker creatine kinase MB (CK-MB), secreted from hepatic and cardiac organoids (Zhang et al., 2017). The changes of charge transfer resistance on an antibody functionalized electrode were measured to quantify the captured biomarkers. After each measurement, a dual-step cleaning process was performed to regenerate the electrode surface, allowing long-term continuous monitoring of these biomarkers. By further integrating two dual-organ platforms with the electrochemical biosensor module, a time-course measurement of drug-induced organ toxicity was demonstrated by a human liver-and-heart-on-a-chip platform insulted with acetaminophen (APAP) for up to 5 days, and a human liver-cancer-and-heart-on-a-chip platform challenged with doxorubicin (DOX) for up to 24 h. This platform showed enormous potential in anticancer drug screening by providing continual sensing of the micro-environmental and biochemical parameters that was not available previously. Despite the capability in continuous biomarker monitoring, these electrochemistry-based TME studies still require sample collection and preparation from on-chip cell cultures to provide the quantitative analysis of the secretory proteins in TOCs. This drawback could be overcome through in situ electrochemical sensors that capture the proteins in proximity to the secretion sites. Liu et al. have demonstrated the first in situ electrochemical sensors that integrate a novel design of half-ring electrodes with a microfluidic cell culture module, to measure the secretory cytokine in a real-time fashion (Liu et al., 2015).

On the other hand, the temporal resolution of electrochemical sensor is greatly limited by the slow analyte diffusion rate over large surface areas on commonly used planar electrodes. Nanoelectrodes exhibit enhanced mass transport and higher current density due to radial diffusion, thus providing not only higher temporal resolution and faster response, but also significantly improved detection sensitivity (Fig. 6A). Nanoelectrodes have been used to detect α -fetoprotein with a detection limit of 0.01 pg/mL (Hong et al., 2020), monitor neurotransmitter secretion at single-vesicle resolution in 0.1 ms intervals (White and Kim, 2021), and map intracellular recordings from 1700 connected neurons (Abbott et al., 2020). The ultrahigh detection sensitivity afforded by nanoelectrodes allowed rapid characterization of cell membrane proteins and secretory chemicals at single-cell level (Liu et al., 2022). In addition, high-throughput electrochemical analysis enabled by electrode arrays has been demonstrated for simultaneous monitoring of isoforms of parathyroid hormone-related peptide (Otieno et al., 2016), and cytokines on wounds (Gao et al., 2021a). With appropriate integration with TOCs, these novel electrochemical techniques could achieve rapid, ultra-sensitive detection of secretory protein in TOCs at high throughput.

5.1.2. Plasmonic biosensors

Plasmonic sensors rely on surface plasmons generated at metal-dielectric boundary, where the refractive index change of surrounding medium leads to spectrum shift of the plasmonic resonance. Therefore, they are sensitive to specific binding events occurring on functionalized sensing surface. Its signal transduction does not require labeled sensing probes and multi-step incubation and washing processes, hence is suitable for in situ real-time detection and monitoring of biomarkers. Surface Plasmon Resonance (SPR) is one of major sensing modalities in

Table 1
Measuring assays applied in TME studies on TOC.

TME components	Specific types	Cancers	Measuring assays	References
Stromal cells	CAFs	Salivary gland adenoid cystic carcinoma	Immunofluorescence, CCD camera	Liu et al. (2010)
	CAFs	Lung adenocarcinoma	Inverted microscope; immunofluorescence; ELISA	Hsu et al. (2011)
	CAFs	Colorectal carcinoma	Immunofluorescence; proteome array analysis	Jeong et al. (2016)
	CAFs	Pancreatic ductal adenocarcinoma	Fluorescent dye; immunofluorescence; live/dead staining; chemiluminescence-based human proteome array analysis	Lee et al. (2018a)
	CAFs	Breast cancer	Phase-contrast image; fluorescent dye; immunofluorescence	Truong et al. (2016)
	CAFs	Breast cancer	Fluorescent dye, apoptosis: Cell-event, fluorescence-based live apoptosis and nuclei stain, flow cytometry	Nguyen et al. (2018)
	CAFs	Pancreatic ductal adenocarcinoma	Luminesce; Immunofluorescence; TPF; AFM; Multiplex ELISA assay; fluorescent nucleic acid stain, fluorescent dye	Lai et al. (2020)
	Human mammary fibroblasts	Mammary	Immunofluorescence; signal harmonic generation (SHG)	Sung et al. (2011)
	Bone marrow stromal cells	Liver tumor	Immunofluorescence; absorbance	Menon et al. (2014)
	Osteo-differentiated human bone marrow-derived mesenchymal stem cells (OD hBM-MSCs)	Breast cancer	Immunofluorescence	Bersini et al. (2014)
Vasculatures	ECs	Breast cancer	Immunofluorescence	Jeon Jessie et al. (2015)
	ECs	Breast cancer	Fluorescent protein; chemiluminescence-based ELISA kit, WB; immunofluorescence; live/dead stain; scRNA-seq	Ma et al. (2020)
	ECs	Breast cancer	Brightfield images; fluorescent dextran; immunofluorescence; fluorescent beads; intrinsic fluorescence of drugs	Aung et al. (2016)
	ECs	Breast cancer	Immunofluorescence; vasculature permeability: FITC-dextran; Cytokine: angiogenesis ELISA	Nagaraju et al. (2018)
	ECs	Kidney cancer	qPCR, beads-based assay (Luminex Assays)	Miller et al. (2018)
	ECs	Glioblastoma	Immunofluorescence	Kim et al. (2013)
	ECs	Leukemia	Immunofluorescence; phase-contrast images; angiogenesis antibody array kit; RT-PCR	Zheng et al. (2016)
	ECs	No real tumor; mimic the presence of pro-angiogenic factors secreted from the tumor	Immunofluorescence, fluorescent protein	Kim et al. (2015)
	ECs	Breast cancer	Fluorescent dextran; fluorescent dye; live/dead staining	Chen et al. (2013)
	TAMs	Metastatic breast cancer	Fluorescent and phase micrograph; SHG, two photon fluorescence (TPF)	Huang et al. (2009)
Immune cells	TAMs	Fibrosarcoma	Fluorescent dye, fluorescent protein; fluorescent dextran; immunofluorescence; bead-based cytokine array	Zervantonakis Ioannis et al. (2012)
	TAMs	Glioblastoma	Fluorescent dye; immunofluorescence; ELISA; flow cytometry; qPCR	Cui et al. (2020)
	TAMs	Lung adenocarcinoma	Immunostaining; ELISA	Hsu et al. (2012)
	Neutrophils	Melanoma	Chemiluminescence-based cytokine array, immunofluorescence; fluorescent dye	Chen Michelle et al. (2018)
	Dendritic cells	Colorectal cancer	Fluorescent dye; flow cytometry; immunofluorescence; qRT-PCR	Parlato et al. (2017)
	Spleen cells	Melanoma	Phase-contrast image; flow cytometry; fluorescence: cell-tracker	Businaro et al. (2013)
	Tumor-infiltrating lymphocyte (TILs)	Colon adenocarcinoma	Fluorescent dye	Moore et al. (2018)
	Tumor fragments including immune cells	Colon adenocarcinoma	Live/dead stain; immunofluorescence; beads-based cytokine profiling; RNA-seq	Aref et al. (2018)
	CXCR4, CXCL12	Salivary gland adenoid cystic carcinoma	Fluorescent dye, immunofluorescence, phase-contrast imaging, fluorescent dextran	Zhang et al. (2012)
	CXCL12, CXCR7, CXCR4	Breast cancer	Western blot (WB), ELISA, qRT-PCR, fluorescent protein, phase-contrast image, bioluminescence	Cavnar et al. (2014)
Non-cellular components	IL-6	Breast cancer	Live/dead staining; fluorescent protein	Mi et al. (2016)
	Wall shear stress	Breast carcinoma	ELISA, qRT-PCR, immunofluorescence	Buchanan et al. (2014)
	Oxygen	Squamous cell carcinoma, lung carcinoma, gastric signet ring carcinoma, breast cancer	Immunofluorescence; microarray analysis, ELISA, qRT-PCR	DelNero et al. (2015)
	Breathing	Lung cancer	Fluorescent protein; absorbance: flow cytometry; immunofluorescence; histology; electrochemiluminescence immunoassay (ECLIA)	Hassell et al. (2017)
	pH, O ₂ , glucose or lactate	Brain cancer	Thin-film platinum, iridium oxide electrodes; electrochemical biosensor	Weltin et al. (2014)
	Cut-off pore size, interstitial fluid pressure	Breast cancer	Immunofluorescence	Kwak et al. (2014)
	Physiological flow	Melanoma	Fluorescence; hematoxylin, eosin and silver staining kit	Albanese et al. (2013)

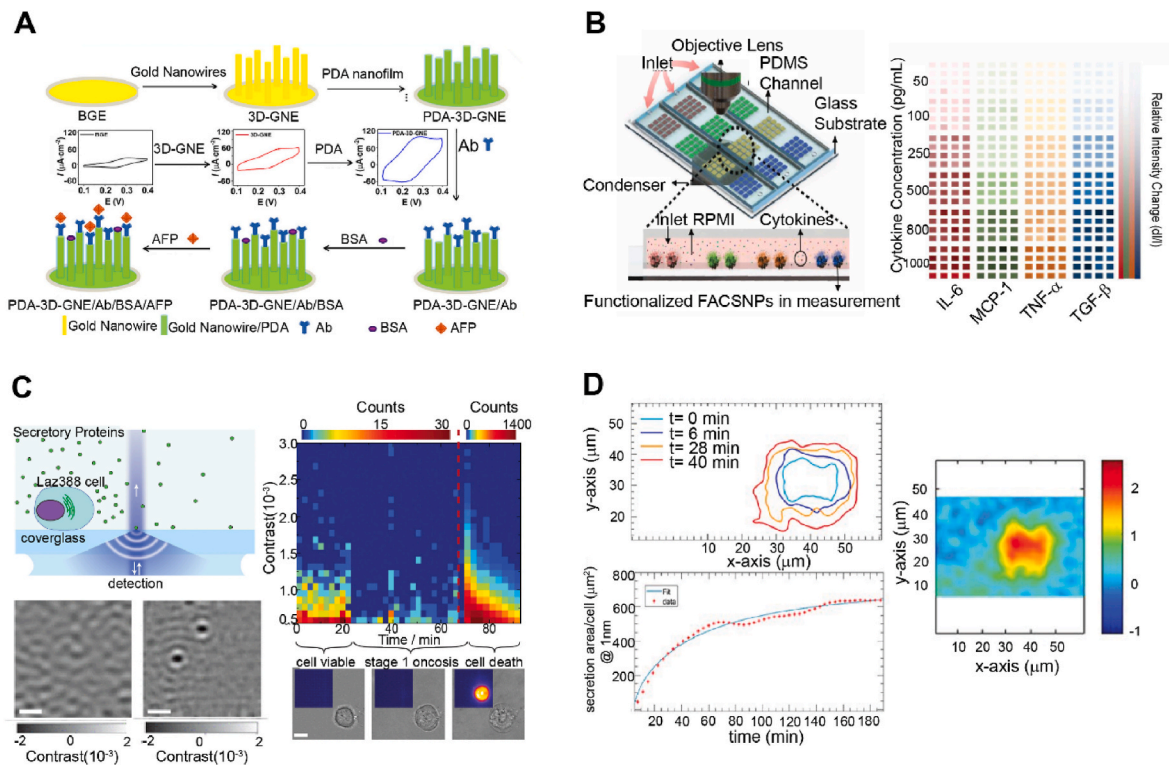


Fig. 6. Secretory protein analysis via real-time detection biosensors. (A) Nanoelectrodes for detection of α -fetoprotein with a detection limit of 0.01 pg/mL. Adapted with permission. Copyright 2020, Elsevier (Hong et al., 2020). (B) multiarrayed LSPR optical immunoassay based on a magnet patterned $\text{Fe}_3\text{O}_4/\text{Au}$ core-shell nanoparticle sensing array for simultaneous real-time detection of four cytokines in complex biological samples, with limit of detection at ~ 20 pg/mL. Adapted with permission. Copyright 2019, Wiley (Cai et al., 2019). (C) Interferometric detection of scattered light (iSCAT) method for in-situ real-time imaging of secreted proteins by Epstein–Barr virus -transformed B cell line. Reprinted adapted with permission from (McDonald et al., 2018). Copyright 2018, American Chemical Society. (D) Photonic crystal resonant (PCR) imaging of thrombopoietin secretion at single-cell level, with fine resolution of 2–6 μm and detection limit below 125 ng/mL. Adapted under the terms of the CC-BY license. Copyright 2018, the Authors (Juan-Colás et al., 2018).

plasmonic sensors that can detect refractive index change in the vicinity of the sensor surface up to 200 nm, thus suitable for label-free detection of large molecule (1–500 kDa) secretion and bulk refractive index measurement. Liu et al. (2012) performed in situ monitoring of vascular endothelial growth factor (VEGF) secretion from human ovarian carcinoma SKOV-3 cells by attaching the cells on the ceiling of the flow chamber, while recording the signals from embedded SPR sensor at the bottom of the flow cell. Compared to SPR, nanoplasmonic biosensors build upon noble nanostructures with local enhancement of the electric field in the vicinity (less than 30 nm) of the surface, yielding a high detection sensitivity for localized binding events. Real-time monitoring of Interleukin 6 (IL-6) secretion at single-cell level was demonstrated by Zhu et al. (2020) using gold-capped nanopillar-structured cyclo-olefin-polymer film with a detection limitation of 10 ng/mL based on spectrum shift measurement. Recently, intensity based nanoplasmonic biosensors have emerged as a new approach that detect the optical responses of scattered light upon target binding, enabling simultaneous multiplex quantification of secretory proteins (Cai et al., 2019; Chen et al., 2015; Faridi et al., 2019; Gao et al., 2021b; Javed et al., 2019; Wang et al., 2021). Cai et al. (2019) developed a high-throughput, label-free, multiplex nanoplasmonic biosensor based on a magnet patterned $\text{Fe}_3\text{O}_4/\text{Au}$ core-shell nanoparticle sensing array (Fig. 6B). This optical plasmonic sensor can achieve real-time, high throughput detection of four cytokines in complex biological samples, with limit of detection at ~ 20 pg/mL. Extraordinary Optical Transmission (EOT) biosensor utilizes periodic nanohole arrays to generate localized and propagating surface plasmons and is another major plasmonic sensing scheme that measures transmission spectra due to refractive index change at surface (Lee et al., 2018b; Soler et al., 2017). Li et al. (2017) developed a microfluidic device combining the cell

culture and EOT sensing modules, thus in situ monitoring of VEGF secreted from cancer cells was achieved at real-time with limit detection of 145 pg/mL.

5.1.3. In-situ real-time imaging

Despite the aforementioned techniques can provide in situ real-time quantification of biomarkers, imaging tools for direct visualization of secretory biomarkers to provide spatial information of cellular secretion in TME have been rare. A few nice attempts have been made using innovative imaging systems that can provide in situ real-time mapping of the secretion profile with micron level resolution. McDonald et al. (2018) reported an interferometric detection of scattered light (iSCAT) method for real-time imaging of secreted proteins from Epstein-Barr virus transformed B cell line (Fig. 6C). The principle was based on the interference of the scattered light produced by the target protein binding with a reference light, which generated iSCAT contrast as the characteristic readout. The hypersensitive real-time imaging of secreted IgG antibodies was accomplished by counting the binding events at different pixels over certain integration period with a throughput of ~ 100 molecules per second. However, the limited sensitivity in detection of low molecular weight molecules (< 100 kDa) hinders its applications for small biomarker (i.e., cytokines and growth factors) monitoring. Juan-Colás et al. (2018) performed secretion mapping of thrombopoietin from individual cells using photonic crystal resonant (PCR) imaging (Fig. 6D). The PCR platform leveraged the constructive or destructive interference of light on photonic crystals (PCs) to monitor the refractive index changes at the surface upon binding with secreted thrombopoietin. The transmission and reflection intensities were recorded for each pixel across a specific range of wavelengths, thus the peak shift can be recognized with a fine resolution of 2–6 μm . Shirasaki et al. (2014)

exploited total internal reflection fluorescence microscopy (TIRFM) for real-time imaging of IL-1 β secreted by monocytes. Fluorophore labeled detection antibodies that bound to IL-1 β in the vicinity of sensor were excited with strong fluorescence emission due to enhanced near-field intensity, which provided high signal-to-background ratio to allow real-time cytokine detection using a single-step incubation at a limit of detection reaching single molecular level.

5.2. Advanced fluorescence molecular imaging for intracellular dynamic biomarker analysis

Fluorescence Molecular Imaging (FMI) has been widely used for *in vivo* analysis of molecular and cellular activities during physiological and pathological processes. FMI resolves molecular features of interest *in vivo* using specific fluorescent probes and reports their presence in a noninvasive fashion. Recent advances in fluorescence microscopic technology have led to the development of novel probes that offer further improved spatial resolution beyond the diffraction limit and ultrahigh sensitivity down to single-molecule level. The previously mentioned fluorescent staining probes used in TME are mostly “always-on” fluorescence probes that provide enhanced contrast signals through accumulation and retention at target binding sites. It often requires a long incubation time for probes to be excreted to generate a high signal-to-noise ratio, limiting their sensitivity and capability in real-time imaging (Fig. 7A). In contrast, novel activatable probes are fluorescently switched on only in response to specific microenvironment indicators and can be interrogated more rapidly upon systemic administration (Zhao et al., 2017). The most common fluorescence switching mechanisms for activatable fluorophores are Förster Resonance Energy Transfer (FRET), self-quenching, and photoinduced electron transfer.

The FRET probes primarily consisted of a fluorophore/quencher pair that conjugate to different sites in vicinity on a probe. The FRET is activated upon target binding that separates the fluorophore/quencher

pair, resulting in images with low background and high activation in designated conditions. Lee et al. (2011) designed a dual-quenched caspase-3-sensitive peptide that achieved 15-fold fluorescence recovery based on caspase-3 activation (Fig. 7B). Since caspase-3 is known as a relevant molecular mechanism in apoptosis signaling, direct visualization of apoptosis in living cells can be achieved through real-time monitoring of the caspase activity. Self-quenching probes adapt FRET principle as well, except that the energy transfer occurs between a ligand protein and an organic dye. Indocyanine green (ICG) has been reported to be effectively quenched by antibody conjugation, and can thus provide fluorescence recovery as high as 50-fold at target site (Nakajima et al., 2011; Watanabe et al., 2014). Photon-induced Electron Transfer (PET) probes, composed of a fluorophore and a redox active receptor ligand, are highly sensitive to pH change or target binding (Urano et al., 2009). Urano et al. (2009) designed acidic pH-activatable probes based on the boron-dipyrromethene fluorophore conjugated to a cancer-targeting antibody. The protonated probe became highly fluorescent with a greater than 300-fold increase in emission in an acidic tumor environment.

Recent advancement in genetic engineering further allows the introduction of reporter genes (i.e., fluorescent protein (FP) gene) in target host cells for *in-situ* imaging of transcriptional regulation, intracellular protein biomarker expression, and protein-protein interactions. By performing a plasmid transfection containing PEG10 promoter and near-infrared protein 682 reporter gene, Shapovalova et al. (2019) analyzed the retrotransposon-derived protein expression via paternally expressed gene 10 (PEG10) in AR-splice variant prostate cancer cells. They discovered that the PEG10 promoter was a powerful and specific tool for noninvasive detection of aggressive prostate cancer subtypes. Thiagarajan et al. (2015) fused a GFP reporter with the promoter of the well-established pluripotency gene NANOG, observing enhanced enrichment for tumorigenic cancer stem cells through real-time GFP imaging. Zou et al. (2017) constructed a red fluorescence protein (RFP)

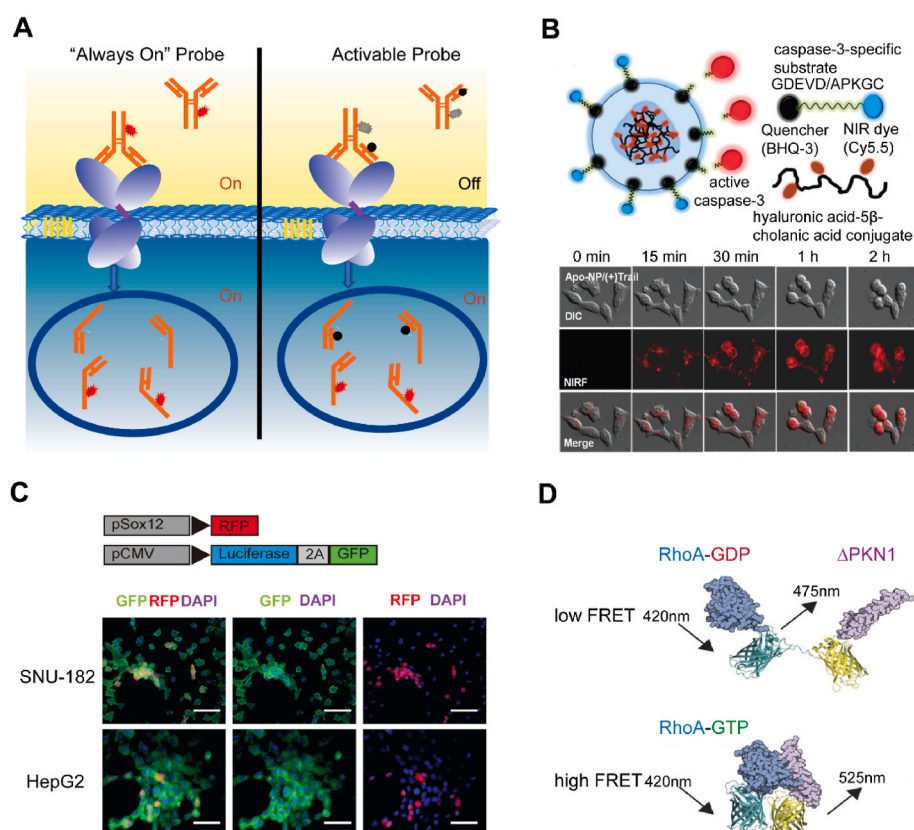


Fig. 7. Fluorescence Molecular Imaging for dynamic biomarker analysis. (A) Principles of “always on” and activatable probe for biomarker recognition. (B) FRET based activatable probes (dual-quenched caspase-3-sensitive peptides) used for monitoring caspase-3 activity. Reprinted adapted with permission from (Lee et al., 2011). Copyright 2011, American Chemical Society. (C) Reporter red fluorescence protein (RFP) gene fused with a Sox12 promoter for the study of Sox12 as a potential cancer stem-like Cell marker in Hepatocellular Carcinoma. Adapted under the terms of the CC-BY license. Copyright 2017, the Authors (Zou et al., 2017). (D) FRET-based FP biosensor used for activity imaging of plasma membrane restricted small GTPase RhoA. Adapted under the terms of the CC-BY license. Copyright 2015, the Authors (Van Unen et al., 2015).

reporter under the control of a Sox12 promoter, to study the function of Sox12 as a potential cancer stem-like Cell marker in Hepatocellular Carcinoma (Fig. 7C). These genetically encoded FPs can be engineered as activatable reporters that are highly sensitive to the intracellular changes of pH, calcium ions (Horikawa et al., 2010) or ATP (Berg et al., 2009) and have been used for dynamic imaging the biological processes within subcellular environments. In addition, more sophisticated strategies by pairing FRET FPs (Fig. 7D) have been applied in intracellular tracking of various biomarkers including metabolites, such as NADH (Zhao and Yang, 2015), glucose (Takanaga et al., 2008), lactate (San Martín et al., 2013), pyruvate (San Martín et al., 2014), glutamin (Marvin et al., 2018), and signaling pathways such as epidermal growth factor receptor (Itoh et al., 2005), Rho family GTPases (Lam et al., 2012; Van Unen et al., 2015), myosin light chain kinase (Khuon et al., 2010), etc. All these advanced molecular imaging probes provide target specific signals with significantly enhanced sensitivity and high tumor-to-background contrast, which can be well integrated as homogeneous sensing and real-time imaging approaches for on-chip analysis of intracellular biomarker, environment and metabolites in the TME.

5.3. On-chip nucleic acid sequencing for genetic and transcriptomic analysis

Nucleic acid sequencing technologies allow for the interrogation of the genetic and transcriptomic information of a tumor and its TME. High-throughput sequencing can identify microenvironmental marker genes and signatures with single-cell resolution that provide genetic fingerprints and spatial information for revealing the intra- and inter-tumor phenotypic and genotypic heterogeneity. In situ nucleic acid sequence tools, in particular for monitoring mRNAs in the TME on-chip, can provide additional information about tumor initiation and progression (Zhao et al., 2020b) and have significant value in prognostics.

5.3.1. Microfluidic assisted high throughput nucleic acid sequencing

The state-of-art high throughput gene sequencing technologies have been widely involved with microfluidics integration for genomic, transcriptomic, and epigenomic single-cell analysis. Four main steps are commonly included in single-cell sequencing: cell enrichment, isolation, lysis, molecular amplification and sequencing. The cell enrichment usually requires identification of specific cellular biomarkers, followed by cell sorting by external force from an actuator to direct them towards desired locations. Many microfluidic cell sorting techniques have been developed, including acoustophoresis (Urbansky et al., 2019), dielectrophoresis (Mazutis et al., 2013), optics (Qi et al., 2017), and magnetophoresis (Blue Martin et al., 2017; Lee et al., 2014). The sorted cells can subsequently be confined in microwells (Manzoor et al., 2021), valve-based microfluidics (Briones et al., 2021), or droplet microfluidics (Ding et al., 2019; Matula et al., 2020) for on-chip lysing. A variety of rapid lysis techniques have thus been reported to minimize the interference with the reactions, such as chemical (Jen et al., 2011), electrical (Jeon et al., 2018; Lo and Lei, 2019), thermal (Privorotskaya et al., 2010) and mechanical lysis (Flaender et al., 2018). The final molecular amplification and sequencing procedures have been mostly accomplished off-chip by various techniques. The details of these techniques have been discussed elsewhere (Hu et al., 2021; Huang et al., 2015; Wang et al., 2022b). Though single-cell sequencing provides powerful means to characterize the TME, the off-chip manipulation inevitably increases the system complexity and overall analysis time and falls short in providing the genomic spatial distribution in tumor organoids (Payne et al., 2021; Wang et al., 2022a). Therefore, several on-chip in situ DNA/RNA sequencing techniques have been explored to fill the gap.

5.3.2. In situ hybridization/sequencing

Instead of extracting DNAs/RNAs from the cells, in situ hybridization/sequencing technology enables direct visualization of these molecules in their original environment. In situ hybridization/sequencing

takes advantage of molecular barcodes, which provide spatial and quantitative gene information without the need for amplification. Barcoding can be fulfilled by inclusion of distinct short DNA oligonucleotides into already prepared cDNA library. Each base of DNA molecules could represent four possible values, thus a short sequence of oligonucleotides can create enormous numbers of combinations for barcoding. The decoding of the barcode integrated with a complimentary probe could then reveal the information of the presence of target genes in situ. The decoding could be accomplished through in situ hybridization (ISH) or in situ sequencing (ISS). The readout in ISH leverages multiple fluorescently labeled probes to hybridize with predefined targets while the readout of ISS is achieved by sequencing-by-ligation (SBL). Xia et al. (2019) developed multiplexed error-robust fluorescence in situ hybridization (MERFISH) to achieve simultaneous measurements of RNA transcripts from ~10,000 genes in individual cells and enable the identification of RNA species enriched in different subcellular compartments (Fig. 8A). Maino et al. (2019) demonstrated an automated microfluidic system for in situ sequencing of barcoded mRNA amplicons in fixed cells (Fig. 8B). This platform was composed of a microfluidic unit, a thermal controller unit, and a reagent delivery unit, allowing automated ligation and hybridization of padlock probes, rolling circle amplification of the barcoded amplicons, and amplicons identification via barcode sequencing. With this system, a higher detection efficiency, shorter protocol time, and lower consumption of oligonucleotide reagents were achieved. Nguyen et al. (2020) successfully performed 3D mapping of 66 genes across 6 chromosomes in hundreds to thousands of cells through SBL. Combining Oligopaints with fluorescence in situ sequencing (FISSEQ), they obviated the need for amplification process and achieved genome-wide super-resolution imaging for human diploid fibroblast cells.

6. The challenges and future of integrated TOC for in situ, spatiotemporal analysis of TME

6.1. Spatiotemporal resolution

Due to the complexity of tumorigenesis and metastasis, tumor cells in the TME can display distinct properties and behaviors at the molecular and genetic levels and evolve rapidly along with the cancer development. This heterogeneity has been found to exhibit high spatiotemporal discrepancy in histology, protein signatures, genetic profiles, and the expression of specific markers. A thorough and comprehensive investigation of these variables places high demands on biosensing technologies that can conduct versatile measurements with unprecedented spatiotemporal resolution on chip. Recent progresses in advanced cancer models including 3D TOC systems have given rise to new challenges for current imaging and sensing platforms by adding another degree of freedom. Thereby, novel engineering tools enabling on-chip homogeneous detection and/or imaging of mechanical forces, chemotaxis, biomarker expression, and bio- and drug distribution are further needed to better understand the complex physiological architecture and gain precise spatiotemporal control of the TME in a 3D approach.

6.2. Non-specific adsorption

The undesired molecular adsorption on polymeric materials, such as PDMS, is a fundamental issue faced by most of the TOC systems, making it extremely challenging to precisely control and monitor the concentration and distribution of the target molecules in the TME. While various novel coatings (i.e., poly (ethylene glycol) (PEG)-, poly-zwitterionic-, saccharide-, polyhydroxy-, amide-containing-hydrophilic-polymers) are being developed to improve the adsorption resistance, the non-specific adsorption remains a technical challenge, particularly for small hydrophobic molecules, due to their strong interactions with the porous PDMS and possible binding onto the coating materials. Similarly, these unintended interactions have also been a critical bottleneck for

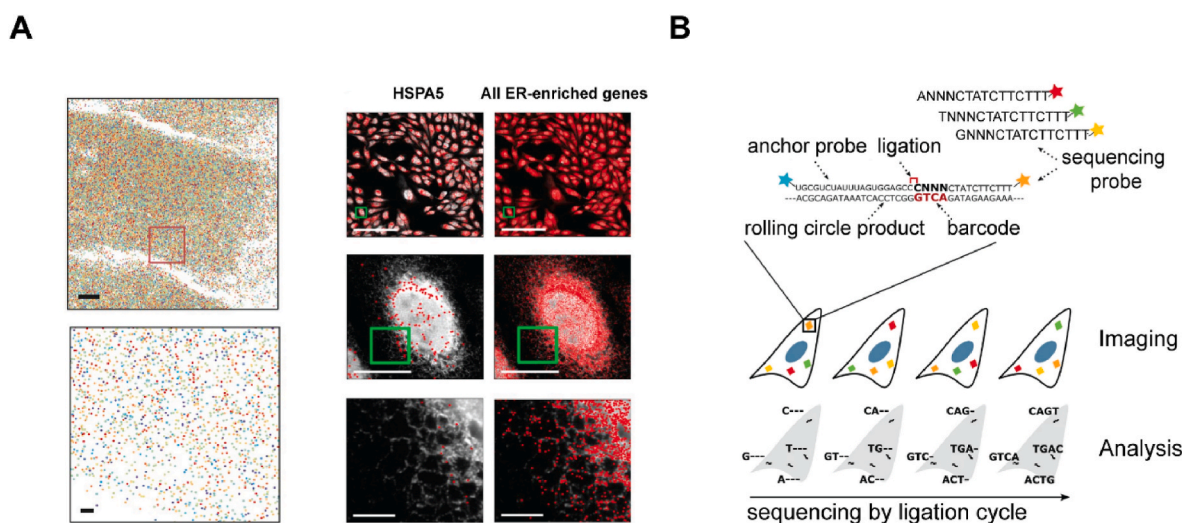


Fig. 8. In situ hybridization/sequencing for TME validation (A) Multiplexed error-robust fluorescence in situ hybridization (MERFISH) for 10,050-gene imaging in individual U-2 OS cells. Left: U-2 OS sample stained with encoding probes targeting 10,050 RNA species and a zoomed-in image below (Scale bar: 1 μ m). Right: identification of RNAs enriched at the endoplasmic reticulum. Adapted under the terms of the CC BY-NC-ND license. Copyright 2020, the Authors (Xia et al., 2019). (B) Schematic for in situ sequencing of barcoded mRNA amplicons in fixed cells. Adapted under the terms of the CC-BY license. Copyright 2019, the Authors (Maïno et al., 2019).

biosensor technologies, especially when integrated with the TOCs for spatiotemporal measurements. Ideally, the real-time, in situ detection of specific molecular binding events in a sea of nonspecific interactions would require either a highly regenerative sensing interface or homogeneous biosensing without the need of separation and washing steps. In-depth fundamental understanding of this phenomenon through theoretical models would also be highly valuable to predict the behaviors of the biomolecules in the TOCs and optimize the sensing performance in complex physiological environments. Such combined experimental and computational efforts would be essential to the development of practical approaches to tackle the nonspecific binding challenge for on-chip sensing and monitoring of the TME.

6.3. High-throughput capacity

Current TOC technologies have shown great potency as predictive preclinical models for cancer research and drug discovery, bridging the gap between animal studies and clinical trials. Though some of the TOCs have demonstrated high-throughput, continuous fabrication of tumor organoids on microfluidics, majority of the models still require labor-intensive and cumbersome processes in preparation, sorting, separation, reagent administration, and characterization. This becomes even more challenging when applying the model systems for high-throughput on-chip analysis of the TME. A recently reported high-throughput TOC platform has exemplified such a system that integrated 192 micro-pumps, 96 individual TOCs, and 384 electrical sensors for high content screening and evaluation of gene expression (Azizgolshani et al., 2021). Nonetheless, unremitting efforts are still needed in sample sparing, device miniaturization and automation, large-scale parallelization, and integration of rapid, highly multiplexed control and sensor systems and massive data acquisition and analysis capability empowered by machine learning, to provide a truly high-throughput TOC platform for on-chip TME analysis.

6.4. Integration and standardization

Though aforementioned TOCs and the biosensing techniques show great promise in modeling and analysis of the molecular traits, growth, and inter-cellular interactions in the dynamic TME, the compatibility to seamlessly connect the workflows and modules of different functions for

an integrated platform has not been well established. This compatibility will involve dedicated efforts to design material interface and flexible and robust sensing interface with high compliance with tumor tissues for real-time, long-term monitoring of the TME. In addition, current TOCs, as an emerging *in vitro* models, lack standardization in cell type, composition, condition, and protocol, which can be technically challenging to create an integrated system with high reproducibility and throughput. Advancements in 3D bioprinting have shown promising features in standardized, large-scale production of fluidic chips for constructing vascularized TME. Further improvement in standardization of TOCs, including the materials selection, device design and manufacturing, workflows, downstream assays, and data acquisition and analysis, will provide robust tumor models for practical applications and open new avenues for patient specific modeling and monitoring systems.

In conclusion, great promises exist in recent advances in TOC devices and biosensing systems for comprehensive characterization of extracellular and intracellular components in TME. The fulfillment of dissection of the complicated TME in TOCs with high spatiotemporal resolution remains challenging that would ultimately require an integrative approach to synergistically combine the viable real-time sensing and monitoring tools with emerging TOC systems (summarized as Fig. 9). Such new technologies capable of accurately observing and manipulating the TME offer immense value in understanding cancer progression and would ultimately gear clinicals with translational diagnostic and therapeutic strategies in cancer treatment.

Author contributions

L.Z., L.L., M.C., and C.M. searched the literature. L.Z. and L.L. prepared the figures. All authors wrote the manuscript. P.C. and W.C. edited the manuscript. All authors commented on and approved the final manuscript.

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.

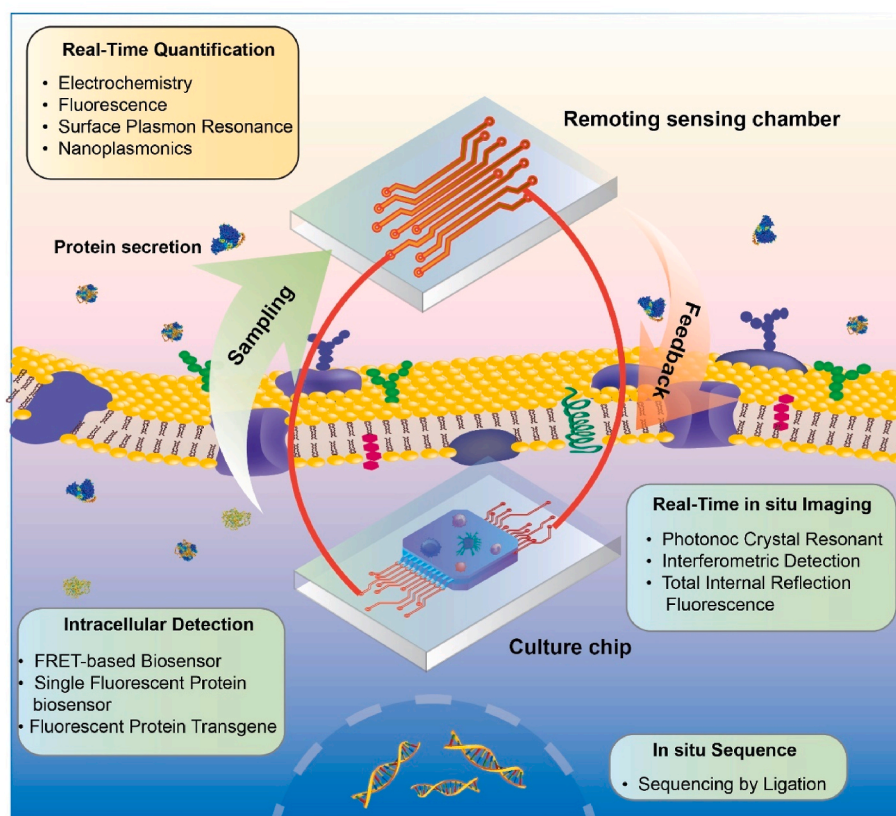


Fig. 9. Conceptual illustration of integrated TOC systems. The integration of viable techniques for real-time sensing and monitoring of TME in TOCs.

Data availability

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

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