Trends in Cancer

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Multiplex Spatial Bioimaging for Combination Therapy Design

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Multiplex spatial analyses dissect the heterogeneous cellular abundances and interactions in tumors. Single-cell bioimaging profiles many disease-associated protein biomarkers in patient biopsies to inform the design of cancer therapies. Guided by the mechanistic insights from spatial cellular maps, combination therapy can efficiently eliminate cancers with reduced off-targets, resistance, and relapse.

Combination therapy is the administration of multiple drugs or modalities for cancer to achieve higher efficacy than using lower doses of individual drugs and to alleviate adverse effects and reduce drug resistance. Despite significant efforts in clinical trials to test combination therapies, the outcome of these therapeutic options varies in individual patients and is associated with treatment failure [1]. While histological inspection is part of a clinical routine in therapeutic planning, multiplexed and single cell analyses of tumors can aid in the mechanistic design of multidrug combinations for clinical applications. To decipher the spatial context of the tumor microenvironment (TME), multiplexed profiling approaches analyze the proteomics, RNA transcripts, metabolites, and epigenetic changes that can reveal the organization of the tumor–immune and tumor–stroma microenvironment, and the heterogeneity among cells. Here, we present the emerging multiplex bioimaging methods that can be used to decipher the synergistic effects of combination therapies on cellular interactions. The current development roadmap of immunotherapies and bacterial treatments that synergistically operate with existing therapies is demonstrated. Multiplex bioimaging of tumor maps provides single-cell mechanisms to decipher the collaborative role of epigenetic, functional, and metabolic mechanisms in combination therapies.

Advent of Multiplex Spatial Analysis

The recent advances in single-cell biotechnologies have provided image-based multiplexing of proteins using either repeated analysis of the cells by fluorescence imaging or multiparameter analysis by imaging mass spectrometry. In these multiplex methods, biopsy samples are sectioned into thin slices, then each tissue piece is labeled by a large panel of antibody libraries, followed by data acquisition by the technology of choice. Single-cell protein analysis methods include Co-Detection by indEXing (CODEX) [2] and cyclic immunofluorescence (CyCIF) [3] that can analyze up to 60-plex biomarkers at a time using sequentially labeled specimens. CODEX and CyCIF allow the monitoring of spatially resolved cell–cell interactions of cancer, immune, and stromal parts in the TME for various cancers at the single-cell level. While the coordination of cell interactions leads to normal biological functions, cancer cells benefit from the multicellular interactions to aggressively grow and therapies bypass these abnormal cellular communications in the TME to effectively eliminate disease progression. Another method for 35-plex proteomic analysis utilizes isotope-labeled antibody libraries that are analyzed by imaging mass cytometry (IMC) [4] and multiplexed ion beam imaging (MIBI) [5], which have been used specifically to study single-cell pathology of breast cancers. Multiplex spatial bioimaging methods provide clues about disease progression and drug response that have great potential to impact the design of emerging therapies.

Trends in Combination Therapies

Combination therapies involve chemotherapy, immunotherapy, radiotherapy, bacterial therapy, and their permutations (see Figure S1 in the supplemental information online). Chemotherapy uses a variety of small molecules that can inhibit cancer growth and invasion. Chemotherapy in combination with immunotherapy might augment, for instance, the acquired immune response by increasing the frequency of cytotoxic T lymphocytes to enhance target-cell destruction efficiency [6]. Multiple immunotherapy methods can be combined to target distinct functional mechanisms. The combination blockade of PD-1/PD-L1 and CTLA-4 checkpoints have been shown to be twice as effective together than either therapy alone [7]. Radiotherapy could also be used in combination with immunotherapy for potential treatments to target metastasis [8]. Bacteria are well suited for anticancer agents because their engineered gene translation machinery produces anticancer proteins and chemotactic receptors that can respond to molecular signals. For instance, local injection of an anti-CD47 immune-checkpoint inhibitor in combination with an engineered *Escherichia coli* strain, releasing tumor-specific anti-CD47 nanobody, promoted the induction of immune responses, increasing tumor-infiltrating T cell activation to enhance tumor regression and prevent metastasis [9]. Carcinogenesis relies on diverse genetic and molecular aberrations, and thus, options for combination therapy can be expanded to cover a wide spectrum of tumor-specific targets that involve signaling proteins, epigenetic biomarkers, and metabolic cues (see Table S1 in the supplemental information online).

Spatial Bioimaging for Combination Therapies

The tumor ecosystem comprises a diverse cellular population. Current combination

therapies are designed based on drug treatments of cell cultures, combinatorial preclinical mouse experiments, and clinical trials in patients. While clinical trials incorporate histological inspection and sequencing of patient biopsies during or after combination therapies, mechanistic insights are still inadequate due to the insufficient details of cellular responses in these multi-drug-testing datasets. Therefore, multiplex bioimaging of tumor samples, through profiling of many protein biomarkers in biopsies, can help determine the effect of synergistic drugs both in the cancer cells and other complementary cells of the TME in individual cells.

Successive spatially resolved single-cell maps of tumors profile biomarkers in a biopsy specimen, which is isolated from a patient during combination therapy at distinct time points. Since these multiplex bioimaging technologies use targeted cellular and molecular profiles of up to, for example, 50-plex molecular signatures, a *priori* information about functional mechanisms of cancer cells and their interactions is needed. This database can be generated based on computational reduction of conventional sequencing and mass spectrometry analysis of tumor biopsies, together with the extensive literature on cellular evidence in combination therapies (Figure 1). Specifically, how potential collective mechanisms can eliminate cancer cells are discussed, and the survival characteristics of the cancer cell such as proliferative signaling, evasion of growth suppressors, resisting cell death, activation of metastasis, replicative immortality, and angiogenesis induction can be targeted.

Immune cells are an essential component in tumor development. A well-established method to determine the cellular composition of the immune compartment of the TME is Immunoscore, a useful tool for classifying the cancer stage. In colon cancer, the density of $CD3^+$ and $CD8^+$ T cell effectors was used as the quantitative metric for Immunoscore to determine the diagnosis or prognosis of a patient [10]. After the introduction of the CD8 cell measurement in cancers, other immune cells [11] including tumor-associated macrophages, myeloid cells, and natural killer cells were studied by single-cell RNA sequencing technologies, which can specifically differentiate cellular differences but lacks information about cellular interactions. These immune markers are unique in each cancer patient, and thus, the spatial maps of the crosstalk between these immune cells in the TME could be the key to precision oncology.

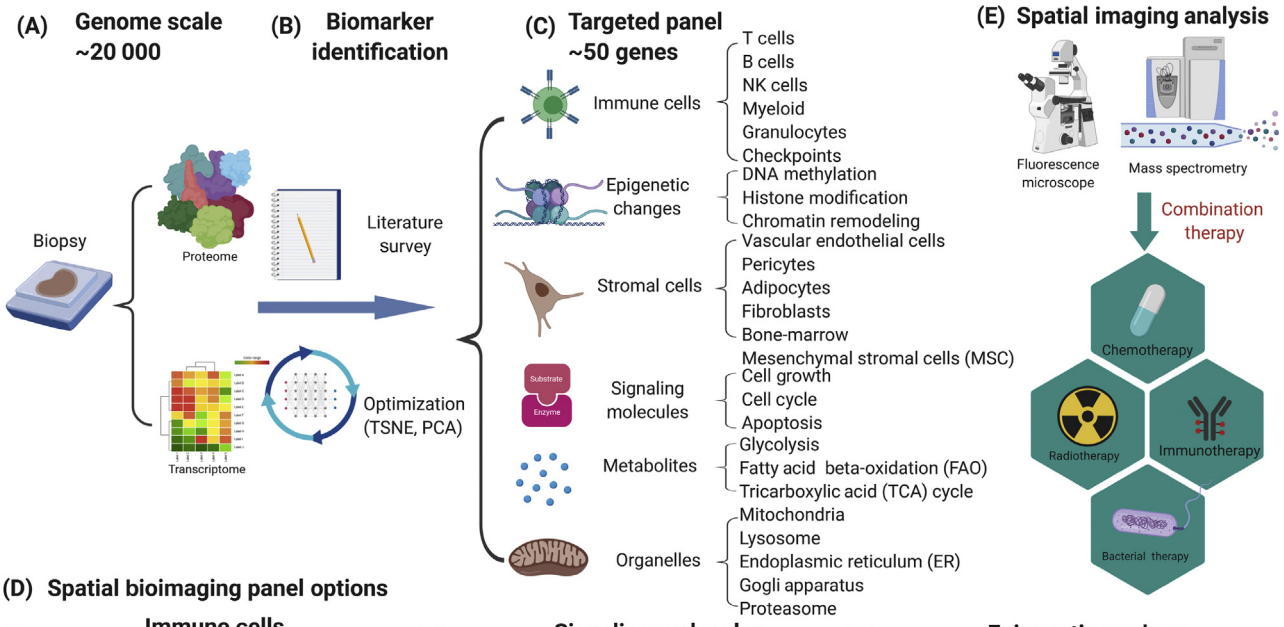
Multiplex profiling can be used to analyze signaling pathways to identify candidate markers for therapeutic resistance. For instance, the multiplex protein imaging method, IMC, utilizes metal-conjugated antibodies to measure protein targets and further predict drug response. IMC provided quantification of the HER2 extracellular domain (ECD), HER2 intracellular domain (ICD), and CD8 markers. A ratio test for ECD: ICD predicted that trastuzumab would be a suitable drug to target the ECD [12]. This finding illustrates the important role of the combination of immunotherapy and chemotherapy in inhibiting the differentiated phenotype of tumors.

Epigenetic changes are a cancer hallmark involved in controlling expression levels of aberrant genes. In cancer, accumulation of DNA methylation inactivates tumor suppressor genes, leading to tumorigenesis. Epigenetic silencing of immunosuppressive genes occurs in precancerous lesions and distal metastatic sites of tumors. For instance, the proteins GSTP1, APC, and RASSF1 undergo epigenetic changes in prostate cancers and have been targeted by multiplex assays as candidate markers for cancer diagnosis and therapy [13]. Epigenetic regulations associated with cancers can be determined from tissue blocks of biopsies using multiplex bioimaging.

Stromal components such as cancer-associated fibroblasts (CAFs) can be targeted in immunotherapies. Thy1, smooth muscle actin (SMA), and fibroblast activation protein (FAP) are candidate biomarkers to predict the effectiveness of anti-PD-1 therapy in metastatic melanoma [14]. Tumor-promoting effects of CAFs and CAF-derived extracellular matrix proteins, enzymes, and chemical factors have been well documented [15]. Therefore, the role of CAFs in inhibiting immune surveillance shows the interaction between CAFs and immune cells, highlighting the potential of developing combination therapy through spatially resolved and multiplex analysis of interactions of CAFs and other cell types in the TME.

The dynamic changes in the cellular metabolism of immune cells can be leveraged for combination therapies. The response of the immune cell to environmental stimuli and the recruitment of other immune cells to destroy invading pathogens provide therapeutic targets to augment antitumor responses. Multiplex ion beam imaging by time-of-flight (MIBI-TOF) is used to analyze the spatially distinct metabolic regulation states of cells that occur in local microenvironments with metabolically remodeled immune cells in diverse tissues [16]. Specifically, MIBI-TOF shows that metabolically repressed cytotoxic T cells, a subset that expresses CD39 and PD1 markers, are excluded from the tumor–immune border in patient biopsies from human colorectal carcinoma.

The communication between organelles is functionally distinct in tumors. In cancer, interactions between mitochondria, endoplasmic reticulum, peroxisomes, and nuclei regulate energy metabolism, biosynthesis, immune response, and cell renewal [17]. Cell culture experiments have validated the synergy of these multiple organelles, but their intricate role in cancer treatment is still underexplored. Since the crosstalk involves multiple biomarker



(D) Spatial bioimaging panel options

(i) Immune cells

CD4	T cell
CD8	T cell
CD19	B cell
CD20	B cell
CD16	NK cell
CD56	NK cell
CD68	Myeloid cell
CD163	Myeloid cell
CD66a	Granulocytes
CD18	Granulocytes
PD-1	Checkpoint
PD-L1	Checkpoint
BTLA	Checkpoint
LAG3	Checkpoint
VISTA	Checkpoint
TIM-3	Checkpoint

(ii) Signaling molecules

mTOR	Cell growth
EGFR	Cell growth
PTEN	Cell growth
MET	Cell growth
Cycline E	Cell cycle
Cyclin D1	Cell cycle
Rb1	Cell cycle
CDK2	Cell cycle
Bcl-2	Apoptosis
Caspase-9	Apoptosis
MYC	Apoptosis
p53	Apoptosis

(iii) Epigenetic markers

GSTP1	DNA methylation
APC	DNA methylation
RASSF1	DNA methylation
H3K27ac	Histone acetylation
H3K4me1	Histone methylation
H3K27me3	Histone mehtylation
MLL3	Chromatin remodeling
MLL4	Chromatin remodeling
UTX	Chromatin remodeling

(iv) Organelles

Mitochondria	Apoptosis
Mitochondria	Bioenergetics
Mitochondria	ROS production
ER	Unfolded protein Response pathway
Golgi apparatus	Modify, sort and package proteins
Lysosome	Proteolysis
Proteosome	Protein degradation

(v) Metabolites

Pyruvic acid	Glycolysis
Glucose	Glycolysis
Acetyl-CoA	FAO
CPT1	FAO
CPT2	FAO
CACT	FAO
Citrate	TCA cycle
Oxaloacetate	TCA cycle
Fumarate	TCA cycle
a-KG	TCA cycle

(vi) Stromal cells

CD31	Endothelial
NG2	Pericytes
PDGFr	Pericytes
CD44	Adipocytes
CD90	Adipocytes
α-SMA	Fibroblast
TnC	Fibroblast
FAP	Fibroblast
CD105	MSC
CD90	MSC

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(See figure legend at the bottom of the next page.)

targets, complex spatial organelle interactions need to be characterized to assess their potential in combination therapies.

Once the targets are identified for combination therapy, the preparation of an experimental multiplex panel is crucial for detecting epigenetic, functional, and metabolic alterations in cancer. To experimentally create a spatial bioimaging panel for multiplex read-out methods, a guideline is presented for combination therapy applications (Figure 2). Although the multiplex assay simultaneously profiles multiple targets, validation of biomarker detection is required to ensure accuracy and reliability. Optimizations are performed for a combination of biomarkers in formalin-fixed paraffin-embedded and fresh frozen biopsy samples using a minimal amount of input samples. Multiplexed and single cell analyses can then demonstrate spatial and cellular distributions and multidrug efficacy.

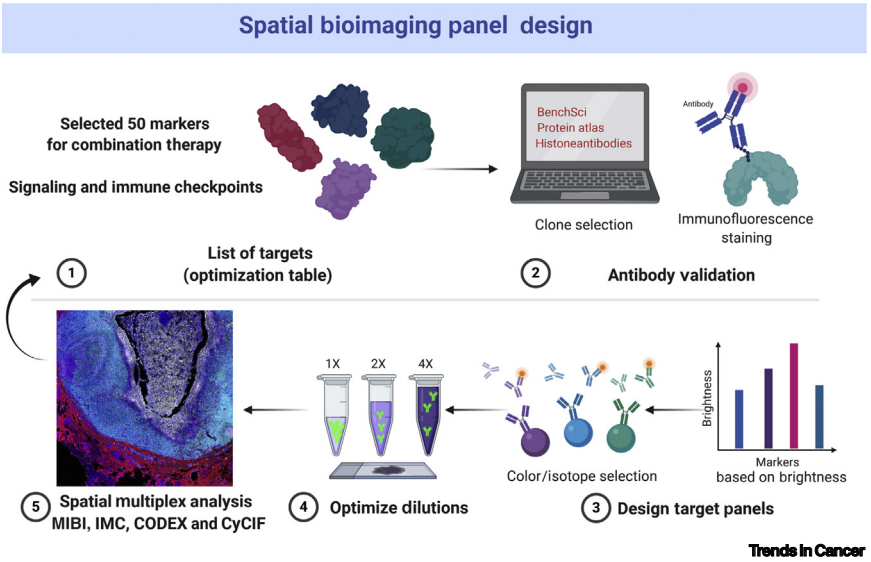

Figure 2. Optimization Strategy for a Multiplex Bioimaging Panel Design. To experimentally reconstruct a spatial bioimaging panel for multiplex profiling methods, step ① is to make a list of selected markers around 50 markers in an optimization table. Step ② is to validate these antibody markers from recent literature and conventional immunofluorescence based on the suggested protocols provided by the antibody vendors. Step ③ is to allocate colors (for fluorescence-based multiplexing) or isotopes (for mass cytometry-based multiplexing) in a multiplexing panel based on the brightness and specificity of each marker. This biomarker assignment procedure balances the dynamic range of labeling concentrations and minimizes the background noise during the multiplex bioimaging experiments. In step ④, the optimal concentration for each marker can be determined by running different dilutions (e.g., 1×, 2×, and 4×) in a sample of interest. Once the optimal concentration is determined, the combination markers can be studied in step ⑤ by multiplex bioimaging methods that include multiplexed ion beam imaging (MIBI), imaging mass cytometry (IMC), Co-Detection by IndeXing (CODEX), and cyclic immunofluorescence (CyCIF) to decipher the spatial context of the tumor microenvironment. A diseased tonsil image of an IMC captured data was shown using a 18-plex optimized multiplex bioimaging panel. Multicolor IMC proteomic image demonstrates pan-keratin (white), CD20 (green), collagen I (red), CD3 (cyan), granzyme-B (yellow), Ki67 (purple), and nuclear DNA (blue), corresponding to distinct cell-specific markers. High Granzyme-B-enriched cellular activity (bright yellow) represents a tonsillar crypt region, surrounded by pan-keratin positive epithelial cells (white edge), as an indicator of disease state in the tonsil. Collagen I (red) shows the extracellular matrix structure of the tonsil. The arrow at ①denotes further optimization of a list of target biomarkers, in which spatial patterns of the candidate markers refine the usability of targets in the design of combination therapies. Created with BioRender.com.

Concluding Remarks

Combination therapy, in which chemotherapy, immunotherapy, radiotherapy, and bacterial therapy mixtures are used, has shown enhanced overall survival rate, better prognosis, and less toxicity. Multiple biomarker modules that cover immune, stromal, epigenetic, metabolites, and organelles could be measured in biopsy samples from patients. Using these multiplex bioimaging data, dissection of the interplay between a set of combination targets to regulate the cell–cell interactions in TMEs is paramount. To enhance the efficacy of emerging immunotherapies in a combination strategy, identification of the synergistic functional targets is needed to induce T cell priming, inhibit the immunosuppressive environment, and reduce the structural complexity of TMEs in cancer. Multiplexing RNA transcripts, proteins, and metabolites of combination targets in bioimaging of TME would be more biologically pertinent for studying functional states of each cell under multidrug

Figure 1. Multiplex Spatial Bioimaging for Combination Therapies. (A) Profiling biopsy samples from patients. Biomarkers are selected from a large amount of genomic or proteomic measurements that can include up to 20 000 targets. (B) To narrow down the targets to ~50 potential markers, the genome-wide data are reduced by optimization methods that include t-Distributed Stochastic Neighbor Embedding (tSNE) and principal component analysis (PCA). A literature survey further supports the selection of key biomarkers for a selected combination therapy design. (C) A multiplexed bioimaging panel covers targets from a subset of distinct categories that comprise immune cells, epigenetic changes, stromal cells, signaling molecules, metabolites, and organelles. (D) A selection of target modules can be selected from (i) immune cells (e.g., CD8, CD19, CD16, CD68, CD18, and PD-1), (ii) signaling molecules (e.g., mTOR, Cyclin E, and p53), (iii) epigenetic markers (e.g., GSTP1, H3K27ac, and MLL3), (iv) organelles (e.g., mitochondria, Golgi apparatus, and lysosomes), (v) metabolites (e.g., pyruvic acid, CPT1, and citrate), and (vi) stromal cells (e.g., CD31, NG2, CD44, TnC, and CD90). (E) Spatial multiplex imaging analysis is performed by an imaging system (a fluorescence microscope or an imaging spectrometry platform) to reveal cell interactions, frequencies, and mechanistic insights for optimized combination therapies that include a permutation of chemotherapy, radiotherapy, immunotherapy, and bacterial therapy. Created with BioRender.com

treatment. Correlation of these spatial omics maps to patient characteristics that include the disease stage, type, demographics, and response to monotherapy could help in the design of personalized treatment plans for identifying precise treatment combinations. Artificial intelligence algorithms could also be integrated with these spatial molecular maps to compute the most synergistic targets from cell–cell interactions toward the development of efficient combination therapies.

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Supplementary Information

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References

1. Wong, C.H. *et al.* (2019) Estimation of clinical trial success rates and related parameters. *Biostatistics* 20, 273–286
2. Goltsev, Y. *et al.* (2018) Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. *Cell* 174, 968–981.e15
3. Lin, J.-R. *et al.* (2018) Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. *eLife* 7, e31657
4. Jackson, H.W. *et al.* (2020) The single-cell pathology landscape of breast cancer. *Nature* 578, 615–620
5. Keren, L. *et al.* (2018) A structured tumor-immune microenvironment in triple negative breast cancer revealed by multiplexed ion beam imaging. *Cell* 174, 1373–1387.e19
6. Ramakrishnan, R. *et al.* (2010) Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *J. Clin. Invest.* 120, 1111–1124
7. Curran, M.A. *et al.* (2010) PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc. Natl. Acad. Sci. U. S. A.* 107, 4275–4280
8. Pitroda, S.P. *et al.* (2019) Integration of radiotherapy and immunotherapy for treatment of oligometastases. *Lancet Oncol.* 20, e434–e442
9. Chowdhury, S. *et al.* (2019) Programmable bacteria induce durable tumor regression and systemic antitumor immunity. *Nat. Med.* 25, 1057–1063
10. Pagès, F. *et al.* (2018) International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* 391, 2128–2139
11. Wu, T. *et al.* (2019) Immune contexture defined by single cell technology for prognosis prediction and immunotherapy guidance in cancer. *Cancer Commun. (Lond.)* 39, 21
12. Carvajal-Hausdorf, D.E. *et al.* (2019) Multiplexed (18-Plex) measurement of signaling targets and cytotoxic T cells in trastuzumab-treated patients using imaging mass cytometry. *Clin. Cancer Res.* 25, 3054–3062
13. Van Neste, L. *et al.* (2012) A tissue biopsy-based epigenetic multiplex PCR assay for prostate cancer detection. *BMC Urol.* 12, 16
14. Wong, P.F. *et al.* (2019) Multiplex quantitative analysis of cancer-associated fibroblasts and immunotherapy outcome in metastatic melanoma. *J. Immunother. Cancer* 7, 194
15. Liu, T. *et al.* (2019) Cancer-associated fibroblasts build and secure the tumor microenvironment. *Front. Cell Dev. Biol.* 7, 60
16. Hartmann, F.J. *et al.* (2020) Multiplexed single-cell metabolic profiles organize the spectrum of cytotoxic human T cells. *bioRxiv.* Published online January 17, 2020. https://doi.org/10.1101/2020.01.17.909796
17. Xia, M. *et al.* (2019) Communication between mitochondria and other organelles: a brand-new perspective on mitochondria in cancer. *Cell Biosci.* 9, 27