Research Techniques Made Simple: Spatial Transcriptomics

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Transcriptome profiling of tissues and single cells facilitates interrogation of gene expression changes within diverse biological contexts. However, spatial information is often lost during tissue homogenization or dissociation. Recent advances in transcriptome profiling preserve the in situ spatial contexts of RNA molecules and together comprise a group of techniques known as spatial transcriptomics (ST), enabling localization of cell types and their associated gene expression within intact tissues. In this paper, we review ST methods; summarize data analysis approaches, including integration with single-cell transcriptomics data; and discuss their applications in dermatologic research. These tools offer a promising avenue toward improving our understanding of niche patterning and cell–cell interactions within heterogeneous tissues that encompass skin homeostasis and disease.

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INTRODUCTION

Rapid advancement in gene expression profiling methods has transformed our ability to understand the tissue alterations associated with development, homeostasis, and disease. The advent of high-throughput next-generation sequencing (NGS) has prompted an omics revolution with exciting technological breakthroughs, such as single-cell RNA sequencing (scRNA-seq), providing novel insights into the cellular heterogeneity of such tissues. ScRNA-seq has enabled the identification of rare cell populations often masked within bulk profiling and pinpointing the expression of genes of interest to specific cell types (Wu et al., 2018). However, scRNA-seq has significant limitations because key spatial information is unobtainable, cells can be difficult to dissociate from archived samples, and dissociation can introduce artifacts in gene expression (Van Den Brink et al., 2017). In addition, cells often coordinate their behaviors as part of niches consisting of multiple and diverse cell types, and lack of spatial information from single-cell assays has limited their biological interpretation in that respect. Addressing this key limitation, the latest omics techniques include a range of methods aimed to quantify gene expression within intact tissues, together known as spatial transcriptomics (ST), which preserve the in situ spatial locations of transcripts expressed within a tissue of interest. ST has the potential to elucidate the coordination of gene expression changes across cell types influenced by their proximity to one another, thereby informing intercellular communication. This article provides an overview of ST methods, principles of analysis for their assayed data, and their applications toward improving our understanding of cutaneous biology and disease.

OVERVIEW OF ST TECHNIQUES

ST encompasses in situ hybridization (ISH), in situ sequencing (ISS), and in situ capturing (ISC) technologies (Figure 1; abbreviations used throughout the text are included in Table 1). These techniques are broadly divided into approaches that target specific genes a priori (ISH and ISS) or capture transcripts in an unbiased manner for transcriptome-wide profiling (ISC). A targeted approach can detect a higher percentage of existing transcripts (i.e., high efficiency) present within the tissue, but fewer unique genes can be assayed at once (typically a few hundred genes, although this number continues to increase). Conversely, unbiased profiling can capture thousands of genes at once but at the cost of lower efficiency (i.e., a fraction of RNA copies for any gene is captured). Thus, each method comes with its own advantages and drawbacks as well as a range of achievable resolutions (Table 2). The resolution of these technologies refers to how exact of a location is retained for any particular transcript and can range from subcellular localization to a 55 μm-diameter capture spot with distinct x–y spatial coordinates. Subcellular approaches can pinpoint transcripts within individual cells and even within subcellular compartments such as the

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Abbreviations: 3D, three-dimensional; ATAC-seq, assay for transposase-accessible chromatin with sequencing; CCI, cell–cell interaction; cSCC, cutaneous squamous cell carcinoma; FISSEQ, fluorescent in situ sequencing; HPRI, high-plex RNA imaging; ISC, in situ capturing; ISH, in situ hybridization; ISS, in situ sequencing; L-lep, lepromatous leprosy; merFISH, multiplexed error-robust fluorescence in situ hybridization; NGS, next-generation sequencing; PCA, principal component analysis; QC, quality control; RR, reversal reaction; RT, reverse transcription; scRNA-seq, single-cell RNA sequencing; seqFISH, sequential fluorescence in situ hybridization; ST, spatial transcriptomics; STARmap, spatially resolved transcript amplicon readout; T-lep, tuberculoid leprosy; t-SNE, t-distributed stochastic neighbor embedding; TH17, T helper type 17; TSK, tumor-specific keratinocyte; UMAP, uniform manifold approximation and projection

SUMMARY POINTS

- Spatial transcriptomics (ST) encompasses a variety of methods aimed to quantify RNA expression directly from intact tissues.
- ST methods include in situ hybridization, in situ sequencing, and in situ capturing technologies, each with its own strengths and limitations.
- ST methods can be combined with single-cell RNA sequencing (scRNA-seq) to maximize the resolution and depth of data.
- Analysis of ST data can localize cell types of interest, identify organizational patterns, that is, niches of cell types within tissues, and reveal potential cell–cell interactions (CCIs).

Advantages

- ST offers high-throughput profiling of hundreds to thousands of genes simultaneously within intact tissue in their native context, preserving the spatial positioning of transcripts.
- Ability to assess the spatial organization of cell types within tissue niches and CCIs.
- Availability of a wide range of computational analysis tools that can also facilitate integration with scRNA-seq data.

Disadvantages

- Each ST method has its own limitations in resolution, capture efficiency, and/or transcriptome coverage, and the burden of choice falls on the investigator.
- Most ST methods are optimized for fresh frozen tissue, whereas adaptation of protocols to formalin-fixed paraffin-embedded tissue has lagged.
- Each tissue of interest may require additional optimization of assay parameters.
- High cost, labor, and/or need for specialized equipment have thus far limited widespread adoption.
- A wide range of analysis methods can be difficult to navigate for finding the method best suited for the investigator's needs.

cytoplasm or nucleus, whereas capture spots that are greater than the diameter of a typical cell (~10 μm) may encompass several cells, obfuscating which exact cell is expressing the transcript. In recent years, these technologies have increasingly shown promise as a viable alternative to scRNA-seq because of their ability to both retrieve RNA information and provide spatial localization and visualization, but all ST technologies currently suffer from the suboptimal depth and/or coverage of the transcripts assayed from intact tissue compared with scRNA-seq. Thus, investigators may find that combining ST with scRNA-seq from the same tissue samples could be synergistic for addressing certain biological questions (Longo et al., 2021).

ISH technologies include subtypes of high-plex RNA imaging (HPRI), such as multiplexed error-robust fluorescence, sequential fluorescence ISH (seqFISH), and seqFISH+ (Chen et al., 2015; Eng et al., 2019; Lubeck et al., 2014; Shah et al., 2016), which differ in their multiplexing capacity (Table 2). ISH techniques allow researchers to directly visualize RNA molecules in their native environment instead of requiring cells to be extracted from tissue and analyzed ex situ. Direct visualization is achieved by hybridizing a fluorescent-labeled probe complementary to a predetermined RNA target of interest (Figure 1a). The signals from the labeled probes are then used to determine quantitative measurements of transcripts in a spatial context. The targeted nature of this approach enables high RNA capture efficiency and single-cell/subcellular resolution of transcripts, with ongoing improvements on the upper limits of the number of targets (Xia et al., 2019). There are also notable disadvantages to ISH technologies because cost and labor significantly increase with the number of targeted readouts, and they require specialized equipment (Asp et al., 2020). Currently, the major drawbacks to ISH technologies are their relative inaccessibility, labor-intensive demands, and constraints on the number of probes (and therefore transcript targets) that can be simultaneously hybridized to the tissue.

ISS technologies, another form of HPRI, include the first ISS protocol using barcoded padlock probes and fluorescent ISS and implement direct base-pair fluorescence readout of cDNA amplicons containing barcodes assigned to known transcripts that are visualized throughout the tissue (Figure 1b) (Ke et al., 2013; Lee et al., 2014; Qian et al., 2020). These approaches also enable subcellular resolution and have the potential to enhance readout to a wider range of targets. The use of barcoded padlock probes further enabled the development of spatially resolved transcript amplicon readout mapping, which improved the efficiency of ISS by bypassing the reverse transcription (RT) step and introduced three-dimensional (3D) localization of transcripts by immobilizing DNA amplicons in a 3D hydrogel (Wang et al., 2018). However, these methods are inherently limited by the need to target known genes and their small fields of view, and several have yet to be demonstrated outside of their originators' laboratories, highlighting their relative inaccessibility.

ISC technologies, in contrast to ISH and ISS, capture transcripts in situ, and sequencing is then completed ex situ, leveraging the massively parallel nature of NGS. This modification is additionally advantageous because it enables unbiased capture of the entire transcriptome. There are a variety of ISC approaches, including 10X Genomics Visium, Slide-seq, and Seq-Scope among others, which differ in their specific methods for capturing transcripts from tissue sections (Cho et al., 2021; Rodriques et al., 2019; Ståhl et al., 2016). However, the general approach of ISC involves placing an array of RT primers each containing distinct positional nucleotide barcodes (assigned to capture spots) and poly-T sequence for mRNA hybridization on slides (Figure 1c). Tissues are sectioned onto these slides and then either fixed, stained, imaged, and permeabilized (Visium and Seq-Scope) or directly hybridized to the barcoded RT primers (Slide-

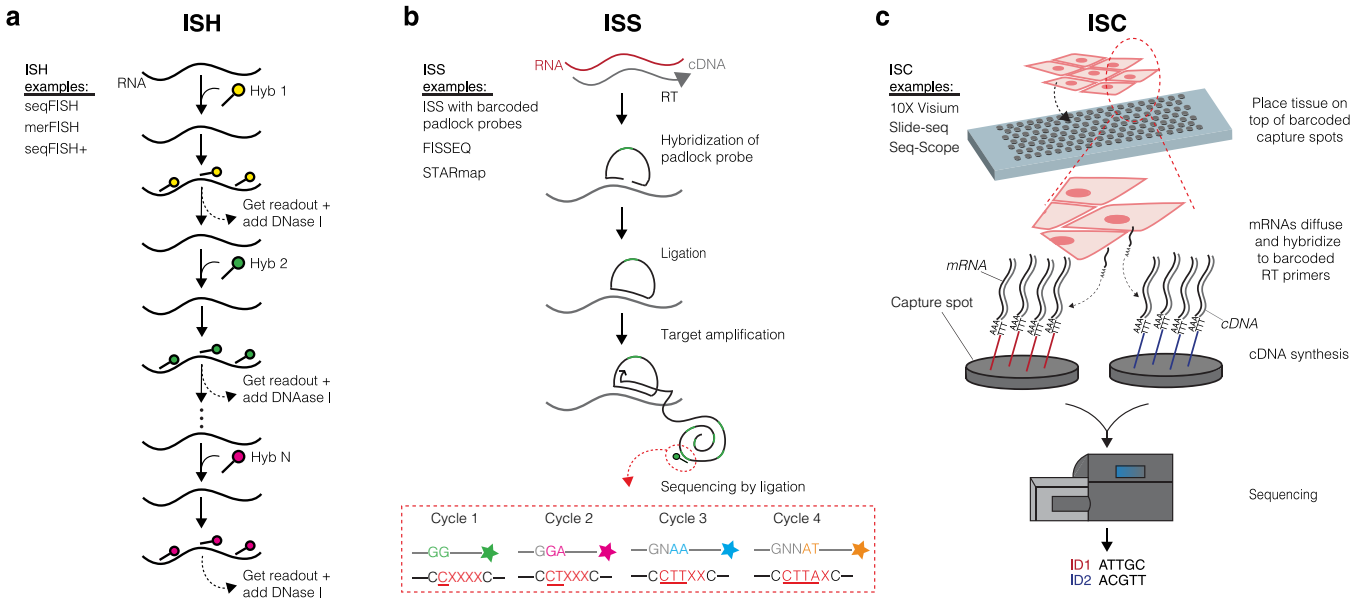

Figure 1. Overview of ST technologies. (**a**) ISH methods detect specific target genes by the use of fluorophore-labeled probes. Signals from probes targeting short sequences of transcripts are propagated by consecutive rounds of hybridization, imaging, and probe stripping. The steps depicted specifically follow the seqFISH methods. (**b**) ISS methods typically involve the hybridization and ligation of a barcoded padlock probe complementary to the RNA or cDNA of a target gene. Multiple rounds of target amplification and sequencing by ligation then allow for spatial resolution of the distinct target gene. The steps depicted specifically follow the ISS with the barcoded padlock probes method. (**c**) ISC methods use capture spots containing an array of RT primers with distinct positional barcodes and poly-T sequences to capture mRNA transcripts. RT produces cDNAs that are extracted and sequenced using next-generation sequencing. Positional barcodes are mapped to specific locations on the tissue and enable spatial visualization of the transcriptome. The steps depicted specifically follow the 10X Visium methods. FISSEQ, fluorescent in situ sequencing; ID, identification; ISC, in situ capturing; ISH, in situ hybridization; ISS, in situ sequencing; merFISH, multiplexed error-robust fluorescence in situ hybridization; RT, reverse transcription; seqFISH, sequential fluorescence in situ hybridization; ST, spatial transcriptomics; STARmap, spatially resolved transcript amplicon readout.

seq). In the Visium workflow, on permeabilization, mRNA molecules from the tissue diffuse downward and hybridize to RT primers. After RT, cDNA is extracted and prepared into sequencing libraries. After sequencing, reads are superimposed back onto the tissue image using the positional barcodes for spatial visualization of the transcriptome (hence, ISC can also be referred to as spatial barcoding). Ståhl et al. (2016) validated their first ISC method (which they named Spatial Transcriptomics, a source of confusion when referencing the various technologies) with mouse brain and human breast cancer tissues. This initial method was later developed into the commercialized 10X Visium technology, which incorporates smaller distances between capture spots and spot diameters reduced to 55 μm for improved resolution. Spot diameter limits resolution because multiple cells are encompassed within the same barcoded regions. ISC methods also currently suffer from lower transcript capture efficiency (i.e., depth) than ISH and ISS in addition to scRNA-seq. Although recent ISC advancements have increased their resolution down to 0.6 and 2 μm-diameter capture spots, one major challenge to overcome will be the tradeoff between RNA capture efficiency and resolution because decreasing spot size diameter typically hinders efficiency (Vickovic et al., 2019).

DATA ANALYSIS FOR ST

Analysis of ST data follows similar principles for all high-dimensional data analysis. In this paper, we cover the steps of ISC data analysis as a model workflow that is similar to those of scRNA-seq analysis and can be summarized into two main phases: preprocessing and downstream analysis (Figure 2). However, although ISH and ISS methods feature unique raw data processing steps associated with converting fluorescence signal into transcript quantification, similar preprocessing and downstream analytical methods can be applied thereafter. The goal of preprocessing is to ensure that high-quality data can flow into downstream analysis, which subsequently seeks to unearth the biological implications of the data. The steps within preprocessing include but are not limited to quality control (QC), normalization, and dimensionality reduction (Figure 2a). QC metrics such as the number of molecules per capture spot (counts per spot) and genes per spot indicate the depth of the data. Normalization accounts for differences in sequencing and capture depth across spots and is further complicated by variations in cellular density across the tissue. The choice of dimensionality reduction method may be based on the priority of two main objectives: summarization (e.g., principal component analysis [PCA]) or visualization (e.g., t-distributed stochastic neighbor embedding [t-SNE] and uniform manifold approximation and projection [UMAP]) (Luecken and Theis, 2019). Currently, limitations for ST methods, namely low transcriptome coverage for HPRI and low RNA capture efficiency for ISC, typically result in less heterogeneity captured within ST data than within scRNA-seq; thus, researchers may find that additional integration with scRNA-seq will yield more value.

After preprocessing, downstream analysis for ST data seeks to identify spatial domains with coherent gene expression, such as tissue niches and cell–cell interactions (CCIs) within,

Table 1. Abbreviations Summarized

Abbreviation	Definition
NGS	Next-generation sequencing
scRNA-seq	Single-cell RNA sequencing
ST	Spatial transcriptomics
ISH	In situ hybridization
ISS	In situ sequencing
ISC	In situ capturing
HPRI	High-plex RNA imaging
merFISH	Multiplexed error-robust fluorescence ISH
seqFISH	Sequential fluorescence ISH
FISSEQ	Fluorescent in situ sequencing
STARmap	Spatially Resolved transcript Amplicon Readout Mapping
CCI	Cell–cell interaction
RT	Reverse transcription
QC	Quality Control
PCA	Principal component analysis
t-SNE	t-distributed stochastic neighbor embedding
UMAP	Uniform manifold approximation and projection
TME	Tumor microenvironment
cSCC	Cutaneous squamous cell carcinoma
TSK	Tumor-specific keratinocyte
CAF	Cancer-associated fibroblast
T-lep	Tuberculoid leprosy
L-lep	Lepromatous leprosy
RR	Reversal reactions
ATAC-seq	Assay for Transposase-Accessible Chromatin with sequencing

or pathogenic domains in diseased tissues where aberrant interactions may occur. This can be accomplished by methods such as clustering as well as those involving integration with scRNA-seq data such as mapping, deconvolution, and ligand–receptor analyses, which informs cell-to-cell communication (Longo et al., 2021) (Figure 2b–d and Table 3). Clustering compares gene expression profiles with groups and identifies similar cell types (if the data are single-cell resolution such as HPRI) or patterns of cell type organization such as tissue layers or niches, which cannot be reconstructed using scRNA-seq alone. Although nonspatial methods of clustering can be applied to ST data, clustering methods directly incorporating spatial information are rapidly advancing. These include methods that account for neighboring spot information, tissue histology, and cellular morphology to improve the recovery of tissue structure from the data (Biancalani et al., 2021; Dries et al., 2021; Hu et al., 2021; Zhao et al., 2021). Mapping and deconvolution integrate scRNA-seq data with spatial data from either HPRI data or ISC data, respectively. Mapping seeks to assign a cell type resolved by scRNA-seq to its spatial counterpart in HPRI data. Deconvolution seeks to predict the proportion of scRNA-seq cell types present within the mixture of transcripts recovered from each capture spot in ISC data, which can convert multicell resolution ISC data to single-cell resolution. Both mapping and deconvolution computational methods are extensively reviewed elsewhere (Longo et al., 2021). A common goal of mapping and deconvolution is to generate cell-type maps that can be used for spatially informed ligand–receptor analyses. These analyses look for statistically significant coexpression of ligands and receptors at proximal locations (such as neighboring cell types) and predict the likelihood of cell communication events by accounting for the effect of distance on gene expression (Cang and Nie, 2020; Dries et al., 2021). Given the spatial restriction of most juxtracrine and paracrine signaling events, spatially informed ligand–receptor analyses can maximize the predictions of CCIs by eliminating CCIs derived from scRNA-seq analyses alone that are spatially implausible. Computational methods for these tasks are quickly evolving, with different methods better suited for different tasks (Lewis et al., 2021). Thus, it is important to keep in mind that several methods may need to be applied to accomplish one's analysis goals.

APPLICATIONS OF ST IN DERMATOLOGIC RESEARCH

Given the diversity of cell types present in normal and diseased skin, ST is poised to become a powerful tool for dissecting the relationships among these various cells. Areas in which ST has proven to be valuable thus far are skin cancer and immune responses in infectious skin diseases (Figure 3). Elucidating ligand–receptor and CCIs in these settings offers the potential for biomarker discovery and therapeutic opportunities. Thus far, ISC technologies have emerged as the most frequently used tool in cutaneous research.

With respect to melanoma research, ST (specifically ISC) was utilized to dissect spatial heterogeneity within lymph node metastases in stage III cutaneous melanoma (Thrane et al., 2018). The authors obtained 2,200 spatial transcriptomes from four patient lymph node biopsies and used PCA and factor analysis to show significant intertumor heterogeneity of spatial expression patterns across patients. Within an individual biopsy, they observed different expression profiles overlapping regions of lymphoid tissue that were either proximal or distant to tumor cells, suggesting a possible influence of the immune composition by tumor cells (Figure 3a). Moreover, this was the very first application of an ISC technology to skin disease.

Another group utilized ST to dissect the tumor microenvironment of cutaneous squamous cell carcinoma (cSCC). They combined ST (ISC, including Visium) and scRNA-seq on a common cohort of patient tumor samples to identify cSCC tumor and stromal cell populations and the spatial niches in which they resided (Figure 3b). They further mapped ligand–receptor networks potentially operating at the leading edge of tumors (Ji et al., 2020). This multimodal approach discovered a tumor-specific keratinocyte (TSK) population localized in a heterogenous manner at the leading edge of tumors, surrounded by a fibrovascular niche, and identified the most likely ligand–receptor pairs engaged in crosstalk among TSKs, cancer-associated fibroblasts, and endothelial cells. The integration of scRNA-seq data with ISC data was essential for prioritizing specific ligand–receptor pairs, some of which were further functionally assessed through in vivo CRISPR screening that identified tumorigenic genes enriched in TSKs and other tumor subpopulations.

ST was also recently utilized to understand the immune response associated with leprosy. Leprosy is histologically characterized by granulomas, organized structures of myeloid cells and lymphocytes surrounding and killing the causative *Mycobacterium leprae* pathogen, and traverses a clinical

Table 2. Comparison of ST Technologies

Assay	Category	Resolution	Capture Approach	Number of Unique Genes Assayed (Multiplex Capacity)	Advantages	Limitations
merFISH	ISH	Subcellular	Targeted	Up to 500 Up to 10,000[1]	Subcellular resolution High RNA capture efficiency	Requires specialized equipment Cost and labor increase with the number of targeted readouts Readout limited to RNA targets
seqFISH	ISH	Subcellular	Targeted	Up to 249	Subcellular resolution High RNA capture efficiency	Requires specialized equipment Cost and labor increase with the number of targeted readouts Readout limited to RNA targets
seqFISH+	ISH	Subcellular	Targeted	Up to 10,000	Subcellular resolution High RNA capture efficiency	Requires specialized equipment Cost and labor increase with the number of targeted readouts Readout limited to RNA targets
Barcoded padlock probe ISS	ISS	Subcellular	Targeted	Up to 100	Subcellular resolution	Limited fields of view Readout limited to RNA targets Lower multiplex capacity than other ISS
FISSEQ	ISS	Subcellular	Unbiased	Whole transcriptome	Subcellular resolution Unbiased readout of the whole transcriptome	Low capture efficiency Difficult to reproduce outside of originators' lab
STARmap	ISS	Subcellular	Targeted	Up to 1,000	Subcellular resolution Increased sensitivity High RNA capture efficiency 3D localization	Limited fields of view Readout limited to RNA targets Difficult to reproduce outside of originators' laboratories
10X Visium	ISC	55-µm diameter capture spots	Unbiased	Whole transcriptome	Unbiased readout of the whole transcriptome Little specialized equipment required	Low capture efficiency Low resolution compared to ISH/ISS
Slide-seq	ISC	10-µm diameter capture spots	Unbiased	Whole transcriptome	Unbiased readout of the whole transcriptome Improved resolution from 10X Visium	Low capture efficiency Low resolution compared to ISH/ISS
Seq-Scope	ISC	~0.6-µm diameter capture spots (subcellular)	Unbiased	Whole transcriptome	Unbiased readout of whole transcriptome Improved resolution and efficiency from 10X Visium and Slide-seq	A small field of view Technical challenges associated with the repurposing of Illumina flow cell

Abbreviations: 3D, three-dimensional; FISSEQ, fluorescent in situ sequencing; ISC, in situ capturing; ISH, in situ hybridization; ISS, in situ sequencing; merFISH, multiplexed error-robust fluorescence in situ hybridization; seqFISH, sequential fluorescence in situ hybridization; ST, spatial transcriptomics; STARmap, spatially resolved transcript amplicon readout.

[1]Demonstrated exclusively in cultured cells; all values without the superscripted number 1 indicate validation in intact tissue samples.

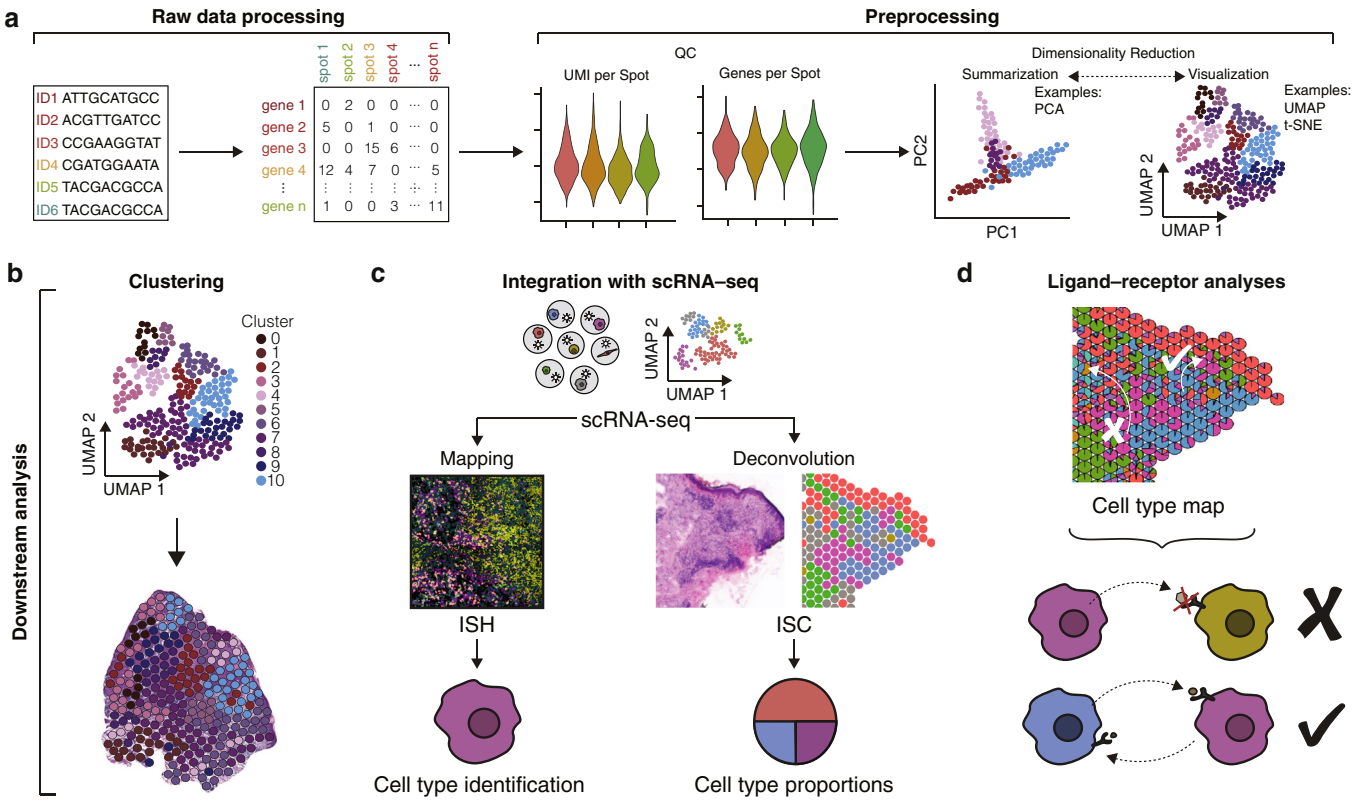

Figure 2. Data analysis workflows. (**a**) For ISC, raw sequencing data are processed into count matrices consisting of genes and capture spots (this step varies depending on ST technique because ISH/ISS involve converting fluorescent imaging signal into similar count matrices). Preprocessing of matrices involves obtaining quality control metrics such as distributions of UMIs per spot (counts per spot) and genes per spot as well as data normalization. Dimensionality reduction methods include summarization methods (PCA) and visualization methods (UMAP and t-SNE), which both seek to reduce gene expression data into fewer dimensions for more informative analysis. (**b**) Clustering groups similar spot transcriptomes, which can be transposed over the original tissue images for general interpretation. (**c**) Mapping and deconvolution combine scRNA-seq data with spatial transcriptomics data to localize cell subpopulations. Mapping typically uses ISH data to localize scRNA-seq profiles and predict specific cell types within the tissue. Deconvolution typically uses ISC data to infer cell-type proportions per capture spot. (**d**) The cell type maps generated from mapping and deconvolution can be applied for ligand–receptor analyses. The proximity of cell types can help infer cell–cell communication events. **c** and **d** were adapted with permission from Ma et al. (2021). ID, identification; ISC, in situ capturing; ISH, in situ hybridization; ISS, in situ sequencing; PC, principal component; PCA, principal component analysis; QC, quality control; scRNA-seq, single-cell RNA sequencing; ST, spatial transcriptomics; t-SNE, t-distributed stochastic neighbor embedding; UMAP, uniform manifold approximation and projection; UMI, unique molecular identifier.

spectrum from limited (tuberculoid leprosy [T-lep]) to disseminated (lepromatous leprosy [L-lep]) disease. A recent study examined patient samples obtained from L-lep, T-lep, and reversal reactions (RRs), during which patients transition from L-lep toward lesions resembling T-lep either through chemotherapy or spontaneously (Figure 3c) (Ma et al., 2021). Utilizing both scRNA-seq and Visium, the authors found that at least two subpopulations of macrophages occupied the central zone of RR granulomas, surrounded by T cells and dendritic cells in the mantle zone periphery. Multiple cell types appeared to contribute to the antimicrobial response in RR, which was driven by IFN-γ and IL-1β produced by lymphocytes (T helper type 17 (TH17) and cytotoxic T cells) and dendritic cells (including Langerhans cells), respectively.

Table 3. Analysis Packages for ST Data

Package	Programming Language	Clustering	Incorporates Histology	Integration with scRNA-seq	CCI Analysis	Additional Features
Seurat	R	Yes	No	Yes	No	Widely used Supports integration with additional single-cell assays (multimodal data support)
Giotto	R	Yes	No	Yes	Yes	Interactive component
BayesSpace	R	Yes	No	No	No	Resolution enhancement of low-resolution data
Tangram	Python	No	Yes	Yes	No	Multimodal data support
SpaOTsc	Python	No	No	Yes	Yes	Infers spatial distance of cell–cell signaling
SpaGCN	Python	Yes	Yes	No	No	Adjustable parameter for weighting of histology on spatial clustering

Abbreviations: CCI, cell–cell interaction; scRNA-seq, single-cell RNA sequencing; ST, spatial transcriptomics.
For a comprehensive overview of data analysis and packages, see Lewis et al. (2021) and Longo et al. (2021).

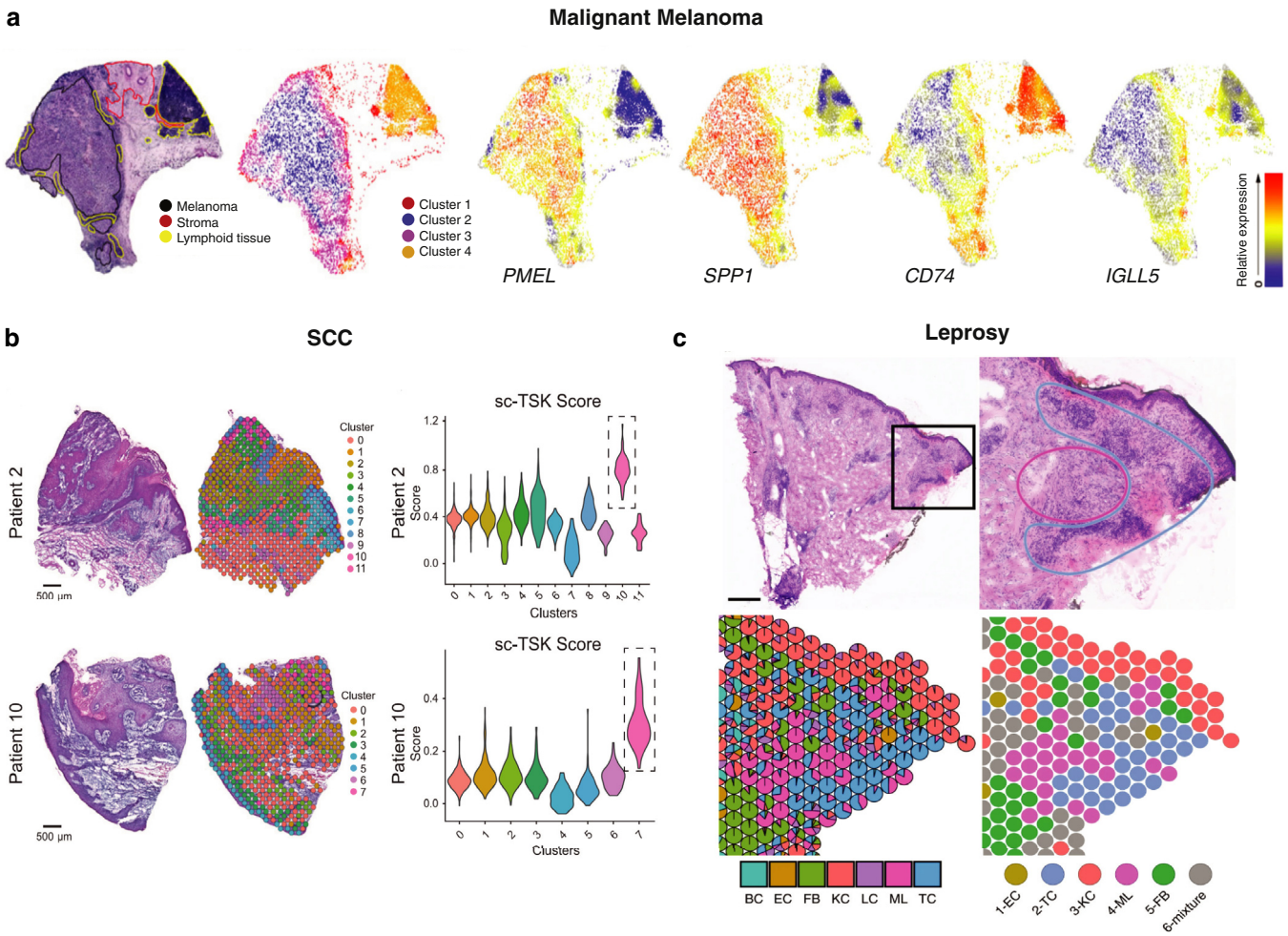

Figure 3. ST applications in dermatologic research. (**a**) ISC from lymph node biopsy of melanoma. Left: H&E staining with pathologic annotation and clustering. Lymphoid tissue occupies two clusters. Right: spatial heatmaps of select highly-expressed and variable genes. Adapted with permission from Thrane et al. (2018). (**b**) Left: H&E staining and clustering of ISC spots in SCC. Right: Violin plots of TSK scores of individual spots derived from scRNA-seq data (sc-TSK score) for each cluster. One spatial cluster within each sample demonstrates a highest sc-TSK score (dotted boxes), highlighting areas occupied by this subpopulation. Reprinted from Ji et al. (2020) under the CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/legalcode). (**c**) Leprosy granuloma architecture and antimicrobial ecosystem. Top: H&E staining of a T-lep biopsy; bottom: cell-type composition map showing MLs in the granuloma center, whereas TCs and FBs occupy the periphery. Bar = 0.5 mm. Reprinted with permission from Ma et al. (2021). BC, B cell; CC, Creative Common; EC, endothelial cell; FB, fibroblast; ISC, in situ capturing; KC, keratinocyte; LC, Langerhans cell; ML, myeloid cell; SCC, squamous cell carcinoma; sc, single cell; scRNA-seq, single-cell RNA sequencing; ST, spatial transcriptomics; TC, T cell; T-lep, tuberculoid leprosy; TSK, tumor-specific keratinocyte.

Thus, ISC enabled the reconstruction of a spatially accurate map of the architecture within leprosy granulomas, allowing for deeper analysis of the contributing factors to the immune response associated with this particular skin disease.

Although several studies mentioned earlier utilized the combination of scRNA-seq and ISC on matched tissues, additional molecular and genomics assays can be integrated to yield further insights. By combining ISC along with lineage tracing and bulk/scRNA-seq and assay for transposase-accessible chromatin with sequencing (ATAC-seq), a recent study characterized wound-associated fibroblast populations in a mouse model of cutaneous wound healing (Foster et al., 2021). They identified four subpopulations of fibroblasts—activated responder, mechanofibrotic, proliferator, and remodeling—and localized them within the wound by applying Visium across various time points for 2 weeks after wounding. Integration of single-cell genomics data with Visium data enabled the imputation of chromatin accessibility changes within fibroblasts through space and time within the healing wound, providing deeper resolution and nuance to the three classical wound-healing stages of inflammation, proliferation, and remodeling. Notably, Visium also identified macrophages in the wound center after 1 week of healing, highlighting additional insights gained from unbiased transcript capture associated with ISC. Thus, the integration of ISC with scRNA-seq and/or other assays can compensate for the currently limited capture efficiency of ISC and facilitate detailed spatiotemporal characterization of a coordinated multicellular and multistate process such as skin wound repair.

CONCLUSIONS AND FUTURE DIRECTIONS

ST can provide a high-dimensional and high-resolution approach to investigating in situ tissue dynamics. Each ST technology has its own tradeoffs in terms of accessibility, transcriptome coverage and depth, field of view, and spatial

MULTIPLE CHOICE QUESTIONS

1. In their current renditions, in situ capturing (ISC) achieves unbiased transcriptome-wide profiling, whereas in situ hybridization and in situ sequencing utilize targeted profiling of a set number of unique genes.
   A. True
   B. False
2. Resolution in spatial assays refers to the following:
   A. The number of transcripts captured per unit area
   B. The number of genes that are represented in the dataset
   C. The x–y positional coordinates of a transcript
   D. The ability to determine a transcript's exact location
3. Which of the following are advantages of ISC over high-plex RNA imaging?
   A. Lower need for specialized equipment
   B. Unbiased capture of transcriptome
   C. Larger field of view
   D. All of the above
4. Which choice best highlights a key advantage of combining single-cell RNA sequencing with spatial transcriptomics (ST)?
   A. Facilitates data collection protocols
   B. Reduces background noise in data
   C. Increases the depth of transcript capture in the data
   D. Quickens preprocessing steps
5. One goal of ST analysis is to do the following:
   A. Improve diagnosis of disease from a patient biopsy
   B. Understand ligand to receptor and cell to cell communication from a spatial perspective
   C. Determine which segments of RNA are eventually translated into proteins
   D. To derive cellular communication strategies based on a person's genetic background

resolution. These factors, along with the specific biological or clinical question, are all considerations when selecting from the various ST assays currently available. Further synergy can be achieved by integrating techniques with deeper transcriptome coverage, such as scRNA-seq, on cells dissociated from the same tissue. Similar to the diversity of ST assays available, a variety of computational methods also exist to perform different tasks for ST data analysis, including clustering, integration with scRNA-seq data, and CCI predictions. In assays where visual information is concurrently obtained, such as H&E staining during the Visium workflow, efforts to incorporate information such as the density or morphology of cells using deep learning approaches continue to emerge (Bergenstråhle et al., 2021; Pham et al., 2020[1]). These methods may help to overcome the current normalization challenges and extract additional insights from these data. Despite the shortcomings of current ST technologies, we envision their broad utility in dermatologic research by elucidating the organization of various cell types present in skin homeostasis and disease and aiding in the characterization of CCIs.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Supervision: ALJ; Writing - Original Draft Preparation: AJP, AEH; Writing - Review and Editing: ALJ

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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DETAILED ANSWERS

1. **In their current renditions, in situ capturing (ISC) achieves unbiased transcriptome-wide profiling, whereas in situ hybridization and in situ sequencing utilize targeted profiling of a set number of unique genes.**

CORRECT ANSWER: A. True

In situ capturing (ISC) leverages poly-T priming to capture poly-A–tailed mRNA transcripts for unbiased transcriptome profiling. In situ hybridization (ISH) utilizes predesigned oligo probes to target predefined transcripts of interest.

2. **Resolution in spatial assays refers to the following:**

CORRECT ANSWER: D. The ability to determine a transcript's exact location.

Resolution in spatial assays refers to the ability to resolve a measured transcript's exact location. Typically, ISH techniques can provide subcellular localization within an individual cell (high resolution), whereas ISC techniques are limited by the diameter of the capture spot (e.g., 55 μm in 10X Visium). Thus, a transcript from ISC can derive from any cell within that particular capture spot.

3. **Which of the following are advantages of ISC over high-plex RNA imaging (HPRI)?**

CORRECT ANSWER: D. All of the above

HPRI encompasses the ISH methods and typically requires specialized equipment for imaging fluorescent signals from probes targeted to the transcripts. In addition, imaging may be limited to a smaller field of view.

4. **Which choice best highlights a key advantage of combining single-cell RNA sequencing with spatial transcriptomics (ST)?**

CORRECT ANSWER: C. Increases the depth of transcript capture in the data.

All ST methods, including both ISC and ISH methods, are unable to measure all available transcripts present within the profiled tissue in terms of coverage and depth. Similarly, single-cell RNA sequencing (scRNA-seq) cannot measure all transcripts present within each cell. Thus, no method can achieve 100% capture efficiency. However, scRNA-seq typically achieves higher capture efficiency and coverage than any ST method alone and can therefore complement ST data.

5. **One goal of ST analysis is to do the following:**

CORRECT ANSWER: B. Understand ligand to receptor and cell to cell communication from a spatial perspective

Given the importance of spatial proximity in cell–cell communication, ST provides the spatial context for the expression of genes associated with communication, such as ligands and receptors, which scRNA-seq is unable to account for.