

Topic introduction

Single-cell and spatial transcriptomic analysis of maize embryo development

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Abstract

Plant embryogenesis defines the biological processes wherein the zygote (fertilized egg) undergoes cell division, cell expansion, and cell differentiation to develop histological tissue layers, meristems, and various organs comprising the primordial body plan of the organism. Studies of embryogenesis in the agronomically important maize crop advances our understanding of the fundamental mechanism of plant development, which upon translation may advance agronomic improvement, somatic embryogenesis, and plant synthetic biology. Maize embryo development is coordinated temporally and **spatially** and is regulated by interactive genetic networks. Single-cell RNA-seq and spatial transcriptomics are powerful tools to examine gene expression patterns and regulatory networks at single-cell resolution and in a spatial context, respectively. Single-cell technology enables profiling of three-dimensional samples with high cellular resolution, but it can be difficult to identify specific cell clusters due to lack of known markers in most plant species. In contrast, spatial transcriptomics provides transcriptomic profiling of discrete **regions** within a sectioned, 2-dimensional sample, although single-cell resolution is typically not obtained and fewer transcripts per cell are detected. In this article, we describe the combined use of these two transcriptomic strategies with synergistic results.

Key words

Single-cell transcriptomic analysis, Spatial transcriptomic analysis, Resolution

INTRODUCTION

Maize (*Zea mays*) is a widely cultivated cereal crop with an overall annual yield of more than 1,161.86 million metric tons (<http://www.worldagriculturalproduction.com/>). Its significance in agricultural and industrial applications is well described (Strable and Scanlon, 2009). Maize embryo development comprises the dynamic, spatial-temporal formation of the first tissues and organs of the plant, although in plants embryogenesis continues throughout the life cycle. As shown in FIGURE 1, after fertilization, the zygote undergoes an asymmetric division to form a small apical cell and a large basal cell, which develop into the embryo proper and suspensor, respectively. The embryo proper establishes both the shoot and root apices, which mediate all subsequent grass development. The non-persistent suspensor is a supporting structure that anchors the embryo proper in the endosperm cavity and enables transfer of nutrients and metabolites to early-staged embryos; at maturity the suspensor is fully degraded (Steeves and Sussex, 1989). The first embryonic organs formed from the maize embryo proper are the shoot apical

meristem (SAM) and the scutellum. The SAM is a pool of stem cells that is ultimately responsible for the development of all the organs in the adult shoot. The scutellum is the first-emerged embryonic lateral organ that serves specialized digestive functions during endosperm digestion and nutrient transfer in the germinating seedling. Subsequently, the coleoptile initiates as the second-emerged embryonic lateral organ, arising from the apical SAM periphery and forming a fused, cylindrical leaf-like organ that entirely encloses the shoot apex. One function of the sheathing coleoptile is to protect the enclosed, underlying seedling (i.e., the SAM and 4-5 leaf primordia) during germination. The third lateral organ to emerge in the grass embryo is the first foliar leaf (L1), which initiates on the germinal side of the SAM. Mature seeds of most maize inbred lines contain 4-5 juvenile leaf primordia, all initiated during embryogenesis in the distichous phyllotactic pattern, where the leaves are arranged in two alternate sides on a stem (Randolph 1936; Abbe and Stein 1954; Poethig et al. 1986; Bommert and Werr 2001; Nardmann and Werr 2009).

The ontology and homology of the grass cotyledon has been a subject of botanical debate for over a century. One model proposed that the scutellum comprises the cotyledon, whereas the coleoptile is a modified foliar leaf (Weatherwax 1920). Another model suggests that the coleoptile is the **cotyledon**, and the scutellum is an evolutionary innovation of the grasses (Boyd 1930). Kaplan (1996) and others proposed a bipartite model wherein the scutellum and coleoptile comprise the distal and proximal domains of the single grass cotyledon. Analyses of the first three embryonic lateral organs generated in the grass embryo provide insight into discussions on the controversial evolutionary-developmental (Evo-Devo) biology of the grass cotyledon, may provide insights into the ontogeny and homology of grass embryonic organs.

Expression of genes implicated in cell differentiation and patterning vary significantly across discrete maize embryonic tissues and organs (Takacs et al. 2012). Previous protocols utilized in comparative transcriptomic analyses of discrete tissues and organs include fluorescence-activated cell sorting (FACS) and laser microdissection-RNAseq (LM-RNAseq). The relative scarcity of transgenic, fluorescent reporter lines have limited the application of FACS in grasses. LM-RNAseq technology enables precise dissection and collection of specific, microscopic, cell types and tissues for gene expression profiling, but does not provide cell-specific resolution. Modern tools such as single-cell RNA-seq (scRNA-seq) and spatial transcriptomic analyses overcome these limitations and reveal cell-specific patterns of gene expression at high-throughput and resolution.

Single-Cell Transcriptomic Analyses

Single-cell transcriptomic analyses, often referred to as scRNA-seq, is a cutting-edge technology that profiles gene expression at single cell resolution. The scRNA-seq protocol comprises five major steps (FIGURE 2): 1) isolation of cells (protoplasts), 2) single cell bar-coding, 3) library construction, 4) RNA sequencing, and 5) data processing and analysis. For plants, the isolation of individual cells requires removal of the plant cell wall and complete dissociation of the resulting protoplasts from undigested plant cells/tissues, using a combination of cell wall-digesting enzymes. The protoplast suspension is loaded into the 10X Genomics Chromium Platform to generate a Gel Bead-In-EMulsion (GEM). The GEM enables the encapsulation of individual protoplasts in microscopic aqueous droplets (microfluidics beads) along with a gel bead containing unique barcoded primers. RNA within each protoplast is captured on the beads, whereafter barcoded cDNA libraries are built following reverse transcription. Each cell-specific cDNA library is then sent for massively parallel sequencing and the resulting data is processed to enable cell clustering and cell-differentiation trajectory projections (pseudotime studies). In single-cell clustering

analyses, most of the significant patterns of variation (i.e. most informative genes) in the data are captured via dimensionality reduction approaches, such as Principal Component Analysis (PCA), followed by visualization of the clusters by Uniform Manifold Approximation and Projection (UMAP). In cell differentiation trajectory projections or pseudotime studies, the algorithms utilize reduced-dimensional data to model the continuous changes in gene expression across cells and then reconstruct the trajectory of the underlying biological process. Once the trajectory is reconstructed, each cell is assigned a pseudotime value. This value represents the position of the cell along the trajectory and estimates its relative stage in the biological process.

The *Arabidopsis* root was among the first and most-studied plant structures using scRNA-seq technology. In 2016, individual *Arabidopsis* root cell protoplasts were first utilized to study stem cell regeneration using scRNA-seq (Efroni et al. 2016). Several subsequent studies focused on *Arabidopsis* root cell types and root developmental trajectory/pseudotime analyses (Denyer et al. 2019; Zhang et al. 2019), abiotic stress responses (Jean-Baptiste et al. 2019), xylem cell differentiation (Turco et al. 2019), and lateral root formation (Gala et al. 2021; Serrano-Ron et al. 2021).

Nelms and Walbot (2019) characterized gene expression programs in single cells comprising the premeiotic stages of male germline development in maize, in the first application of scRNA-seq analysis in grasses. The study found that nearly all pre-germinal cells proliferate, which fails to support a stem-cell model to generate meiotic cells. Subsequently, Satterlee et al. (2020) profiled the transcriptome and inferred the cell differentiation trajectory of the maize shoot apex at single cell resolution. Pseudotime analyses revealed that ectopic expression of *KNOTTED1* (*KNI*) accelerates cell differentiation in maize leaves by promoting sheath development. Subsequently Liu et al. (2021) profiled the single-cell transcriptome of root tips in rice and identified novel cell types associated with phytohormone biosynthesis, signaling, and response. Later scRNA-seq profiled cell-specific gene expression and differentiation of additional grass organs, including maize and *Setaria* roots (Ortiz-Ramirez et al. 2021), leaves and roots in rice (Wang et al. 2021; Zhang et al. 2021), the maize female inflorescence (Xu et al. 2021), the rice flower (Zong et al. 2022) and maize seedling leaves (Satterlee et al. 2023). These studies demonstrate how the application of scRNA-seq technology can deliver broad insights into plant development and differentiation via the detection of novel cell types and the construction of gene expression landscapes across diverse **tissue** types. Moreover, scRNA-seq facilitates the tracking of single-cell differentiation over developmental time via pseudotime analyses that are not possible using conventional, bulk-RNAseq.

Spatial Transcriptomic Analyses

One drawback to conventional scRNAseq analyses of plants is that the protoplasting technology removes cells from their spatial context within the plant. As a consequence, prior (or subsequent) studies of cell/tissue-specific gene expression are required to identify the original, within-the-plant location of each cell analyzed in a scRNA-seq experiment. In contrast, spatial transcriptomics enables gene expression profiling while preserving the spatial context of the cells, tissues, and organs analyzed. Two major strategies for spatial transcriptomic methods are currently available, including fluorescence *in situ* hybridization (FISH)-based and high-throughput sequencing-based studies. FISH-based spatial transcriptomic approaches utilize multiplex fluorescence *in situ* hybridization (M-FISH) techniques, wherein multiple gene-specific probes utilizing different fluorophores bind to the native transcripts within cells/**tissue and** enables expression analyses of multiple genes simultaneously (Moffitt et al. 2016). Although M-FISH protocols are commercially available (Laureyns et al. 2022), high-throughput analyses

are currently unavailable and thus will not be further-discussed in this short review. Sequence-based spatial transcriptomic protocols utilize universal poly(dT) probes to capture transcripts that are subsequently reverse-transcribed, amplified, sequenced, while spatial-barcodes identify the tissue-specific locations of these transcripts (Stahl et al. 2016). Specifically, spatial-barcodes comprise specific probe sequences for every capture spot on the sample slide, thereby delivering spatial information for each transcript detected.

High-throughput sequencing-based spatial transcriptomic methods feature six major steps (FIGURE 3): 1) sample collection and histological sectioning, 2) tissue optimization (optional) and tissue permeabilization, 3) library construction, 4) RNA sequencing and 5) data processing and analysis. Most current spatial transcriptomic platform, such as 10X Genomics Visium™, Slide-seq, and Stereo-seq, require fresh-frozen samples embedded in an optimal cutting temperature (OCT) reagent to maintain RNA quality, whereafter samples are cryo-sectioned, slide-mounted and stained (Rodrigues et al. 2019; Fawcner-Corbett et al. 2021; Wei et al. 2022). Sample slides are pre-printed with multiple universal probes containing spatial-barcode information as well as unique molecular identifiers (UMIs), thereby yielding spatial information while also labelling every individual, captured, RNA molecule. Probes may be **allotted** into RNA capture-spot arrays or bound to beads immobilized on the slides; probes on each individual capture-spot (or bead) share the same spatial-barcodes. Tissues are then permeabilized to release RNA molecules from the tissue sections; mRNAs then hybridize to the underlying probes within the beads/spots. Tissue permeabilization time is a critical parameter affecting data quality. If the permeabilization time is too short, insufficient amounts of RNA may be released from the tissue, causing data loss. In contrast, if the permeabilization time is too long, the released RNA may diffuse to adjacent geographic locations, resulting in errant spatial information. As such, the tissue-permeabilization time must be optimized when processing any tissue/organ/species for the first time. The released RNA is then reverse transcribed into cDNA, then amplified prior to library construction. Since the slide-bound probes are used for reverse-transcription, each cDNA molecule contains a spatial-barcode and a UMI. Resultant libraries are then sent for sequencing, and the resulting data is mapped to the corresponding geographic location of the tissue-sections utilizing spatial-barcode data. In this way, gene expression data is correlated to geographic locations containing specific cells/tissues/organs.

The Visium spatial-transcriptomic platform is most-often utilized in animal studies (Tirado-Lee 2021), but was first utilized in plants in studies of *Arabidopsis* inflorescence meristems, leaf buds in *Populus tremula*, and female cone development in *Picea abies* (Giacomello et al. 2017; Giacomello and Lundeberg 2018). A modified Visium protocol was used in our recent, spatial transcriptomic study of maize embryo development (Wu et al. 2024, *bioRxiv*). Another newly-emerged spatial transcriptomic protocol termed "Stereo-seq" combines nanoball array chips (chips on a slide coated with densely packed probe nanoballs that carry spatial barcodes) and *in situ* RNA capture to achieve transcriptomic analyses at subcellular-level resolution (Liu et al. 2022; Xia et al. 2022).

Several features of plant cells present challenges to their use in spatial transcriptomics. Many plant cells contain large, water-filled vacuoles that readily form ice crystals upon freezing; when cryosectioned, these ice-filled vacuoles may destroy both subcellular structures and tissue integrity. Likewise, the cell wall inhibits permeabilization of RNA molecules to the underlying slide beads, such that ultra-thin tissue sections (i.e. less than one cell diameter thick) or cell wall digestion may be required to enable tissue permeabilization. Once these challenges are overcome, spatial transcriptomics are powerful tools providing *in situ* transcriptomic information for plant cells.

Integration of Single-Cell RNAseq and Spatial Transcriptomic Approaches

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Although single-cell RNA sequencing and spatial-transcriptomics are cutting-edge technologies to identify specific cell clusters and infer cell differentiation trajectories in plant tissues, each technique has advantages and limitations that are summarized in Table 1. For example, scRNA-seq profiles the transcriptomes of three-dimensional samples at single-cell **resolution** and may detect several thousand gene transcripts for each of several thousand cells (Satterlee et al. 2020; Shahan et al. 2022; Satterlee et al. 2023). Such large datasets boast statistical power, data generalizability, and reduced sample bias, all of which aid in the identification of rare cell types and low level transcripts. However, scRNA-seq does present complications specific to plant cells. Plant tissues contain cells of variable sizes and cell-wall architectures, which may introduce cell-type biases after protoplast digestion. For instance, some plant cells contain secondary cell walls that may hinder protoplast digestion, whereas extra-large cells may block the microfluidic processes generating the GEM, resulting in the absence of certain cell types from the datasets. In such cases, researchers may use single nucleus RNA-seq (snRNA-seq) as an alternative, although nuclei typically yield far less RNA than a whole protoplast (Bakken et al. 2018; Selewa et al. 2020; Chen et al. 2023). In addition, an abundance of cell/tissue-specific marker genes is unavailable for many plant species, presenting problems in identification of the tissue-specific origins of cell clusters. In such cases, researchers must conduct a series of time-consuming and labor intensive analyses to identify cell cluster-specific marker genes.

Spatial-transcriptomic approaches partially compensate for the limitations of scRNA-seq procedures. Cryosectioning preserves RNA quality, thereby minimizing the risk of RNA degradation. Moreover, spatial-transcriptomic approaches present fewer limitations on cell size, although this may be a factor influencing RNA permeability. Cell wall properties may likewise hinder RNA permeabilization, although adjusting the thickness of the cryosectioned samples may reduce this problem. Critically, spatial transcriptomics yields gene expression data while preserving the geospatial context, thereby assisting the identification of cell clusters.

Among the noted limitations of spatial transcriptomic methods is the so-called resolution-gene detection dilemma. Giacomello et al., (2017) utilized spatial transcriptomic slides featuring capture-spots of 100 μm diameter, where the center-to-center distance was 200 μm (i.e. a 100 μm gap between two adjacent spots). Maize shoot meristematic cells are ~ 10 μm in diameter, such that the capture-spot area and the gap area may include 10 or more cells. This relatively low resolution may reduce the efficiency of spatially locating transcripts from smaller cells/tissues and young organ primordia. Recently, 10X Visium doubled the bead resolution of their spatial transcriptomic slide platform, although the system still struggles to parse signals from closely-packed tissues or organs. For example, the maize embryonic SAM, coleoptile, and leaf are closely positioned within a 150-200 μm circle area, so that a Visium capture-spot may encompass multiple organs (FIGURE 4). Slide-seq and Stereo-seq protocols **feature improved resolution** of up to 10 μm and 500 nm, respectively, thereby exceeding single-cellular or subcellular resolution (Yu et al. 2023). However, one drawback to this higher resolution is a dramatic reduction in the sensitivity of transcript detection, defined as the percentage of **detected unique transcripts relative to the total number of unique transcripts in a particular area** (Stahl et al. 2016; Asp et al. 2020). BGI Genomics asserts that Stereo-seq is able to detect greater than 500 genes per cell (BGI website: <https://www.bgi.com/global>), although no demonstrations have been reported in plant research.

A second limitation is that data gathered from spatial-transcriptomic approaches may fail to represent the entire, three-dimensional sample of interest. Cells may not be distributed or arranged homogeneously within a tissue sample, and different sectional planes may reveal distinct cell-type profiles. For example, a transverse section through the maize embryo may visualize the SAM but can miss the root pole. Therefore, it may be necessary to process multiple sections from different planar axes in order to sample

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cells from these different tissues. The application of spatial-transcriptomic protocols in high-throughput, on complete serial-sections of three-dimensional samples, is not yet practical.

CONCLUSION

Owing to their relatively small vacuoles and thin cell walls, maize embryonic cells are ideal for single-cell RNA-seq and spatial-transcriptomic research. To overcome the specific limitations currently inherent to each of these two protocols, we combined the two analyses in an integrated approach that enhanced our understanding of the spatial organization and expression landscapes of individual cells in this complex biological system. In this review, we suggest that scRNA-seq analyses provide the majority of the cell-specific transcriptomic data, whereas the spatial-transcriptomic procedures were critical to the verification of cell cluster identities. When selecting from among the variety of currently-available spatial-transcriptomic protocols, researchers must strike a balance between limitations in gene detection versus spatial resolution.

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COMPETING INTEREST STATEMENT

The authors report no competing interests

AUTHOR CONTRIBUTIONS

H. Wu wrote the manuscript and generated all the Figures and sample data. M.J. Scanlon edited the document.

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TABLE 1 Advantages and limitations of the single cell and spatial transcriptomics approaches

	Advantages	Limitations
Single cell RNA-seq	1) Profile three-dimensional samples 2) Single-cell resolution 3) High gene detection sensitivity (5-40%*)	1) Cell wall digestion variation may cause cell type bias 2) Extra-large cell may block the emulsion process 3) Difficult to identify cell clusters due to lack of known markers in most plant species
Spatial transcriptomics - Visium	1) Spatially-resolved transcriptome profiling 2) Good RNA integrity 3) Cell size is not a limiting factor 4) High gene detection sensitivity (>6.9%*)	1) Profile two-dimensional sections 2) Low resolution (5-10 cells)
Spatial transcriptomics - Slide-seq and Stereo-seq	1) Spatially-resolved transcriptome profiling 2) Good RNA integrity 3) Cell size is not a limiting factor 4) High resolution (single cell or subcellular)	1) Profile two-dimensional sections 2) Low gene detection sensitivity (0.3%*)

* The gene detection sensitivity is defined as the percentage of detected unique transcripts relative to the total number of unique transcripts in a particular area. Data from Asp et al. (2020)

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Figure legends

FIGURE 1 General procedure of maize embryogenesis until the Leaf1 stage

- Proembryo stage. After fertilization, the zygote undergoes asymmetric cell division to form a small apical cell and a larger basal cell, which develop into the embryo proper and suspensor, respectively.
 - Transition stage. The embryo proper cells differentiate a shoot apical meristem (SAM) on its germinal face and the scutellum on the abgerminal side.
 - Coleoptilar stage. The coleoptile initiates above the SAM adjacent to the scutellum
 - Leaf1 stage. The first leaf primordium initiates from the SAM. Cells below the SAM differentiate into the hypocotyl and root apex. Vasculture tissue becomes histologically visible in the scutellum.
- The figure was adapted from Wu *et al.* 2024b.

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FIGURE 2 The procedure of single-cell transcriptomic analysis.

- Maize embryos are dissected from developing kernels: cell walls are digested in the digesting solution, followed by isolation of these **resulting** protoplasts.
- Protoplasts are mixed with 10x barcoded gel beads and embedded in oil, forming a Gel Bead-In-Emulsion (GEM).
- Single cell GEMs are collected; cDNA is synthesized through reverse transcription (RT). cDNA strands are then pooled, followed by oil removal. The cDNA molecules are amplified for library construction.
- The cDNA library is sent to be sequenced.

- E. The read data is processed for subsequent analyses. The example graph shows a Uniform Manifold Approximation and Projection (UMAP) layout with 11 cell clusters (cluster 0 through 10 coded by different colors).

Step B and C are adapted from the 10x Genomics website

(<https://www.10xgenomics.com/products/single-cell-gene-expression>)

Commented [A8]: Do you want to discuss what is actually shown in this graph as an example?

Commented [MOU8R2]: Added

FIGURE 3 The procedure of spatial transcriptomic analysis.

- The developing kernel is cut longitudinally, and the mid-kernel region containing the embryo is retained. The mid-kernel region is fresh-frozen in optimal cutting temperature (OCT) and sectioned using a cryostat.
- Tissue sections are mounted on the capture areas of spatial-transcriptomics slides. When processing a tissue or organ for the first time, it is highly recommended to perform a tissue optimization step to determine the optimal permeabilization time. During tissue permeabilization, RNA is released from the section and binds to probes on capture spots.
- First-strand cDNA is synthesized via reverse transcription (RT), followed by second strand cDNA synthesis. The double stranded cDNA molecules are then released from the slides and undergo amplification for library construction.
- The cDNA library is sent to be sequenced.
- The read data is processed and mapped to the sections based on the spatial barcode.

Step B is adapted from the 10x Genomics website (<https://www.10xgenomics.com/products/single-cell-gene-expression>)

Commented [MOU9]: Tissue optimization has been described in detail in the text, so I deleted the technical detail here.

Commented [MOU9R2]: Thanks.

FIGURE 4 Maize embryo overlaid with the Visium capture spots

The red circles are capture spots covered by the embryo. The shoot apical meristem (SAM), upper and lower coleoptile, and the leaf are located within the blue-circled area. Scale bar = 100 μ m.

Protocol

Single-cell and spatial transcriptomic analysis of maize embryo development:

A sample preparation protocol

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ABSTRACT

Maize is an important crop that contributes to the modern food economy in various ways, including human consumption, animal feed, and industrial products. Maize research provide valuable insight into maize improvement and may stimulate local/national economies while facing the food demands of a growing population. As the primordial stage of the corn life cycle, maize embryogenesis determines the fundamental body plan and developmental programs that organize the tissue patterning and subsequent growth and reproduction of the corn plant. Investigating maize embryogenesis at high, cellular resolution can enhance our understanding of the homology, ontogeny, and developmental genetic mechanisms of embryonic organ morphogenesis. However, no studies have yet described methods for analyzing maize embryo development at single-cell resolution. Single-cell RNA sequencing (scRNA-seq) and spatial transcriptomic analyses are powerful, combinatorial tools toward studying maize embryogenesis at the single-cell level within a spatial context. These methods can reveal transcriptomic relationships between tissues/organs and provides insight into the gene regulatory networks operating during embryogenesis. In this protocol, we describe a detailed procedure to prepare maize embryo samples for construction of scRNA-seq and VisiumTM spatial transcriptomic libraries that are suitable for massively parallel sequencing. Our protocol borrows from prior published studies and manufacturer's instructions and is optimized to adapt to studies of the maize embryo.

Introduction

Maize (*Zea mays*) is an important cereal crop and has wide applications in agriculture, industry, and scientific research. As a model system of monocot grass, maize plays a crucial role in grass molecular and developmental biology studies. Grass embryo development is a complex process that leads to the formation of diverse tissues and organs. Single-cell and spatial transcriptomic techniques are powerful tools that can be used to investigate plant ontogeny at high resolution and in high throughput, although these approaches have not yet been applied to grass embryogenesis studies.

Single-cell transcriptomic techniques analyze the gene expression of individual cells, allowing novel insight into tissue/organ development at a single-cell resolution. These protocols utilize microfluidic systems to prepare cell-specific transcriptomic libraries. Cell-specific transcripts are captured by beads with barcoded oligonucleotide probes that mark individual cells and transcripts, followed by reverse transcription (RT), cDNA amplification, library preparation, and high throughput sequencing to extract cell-specific gene expression profiles.

Commented [A10]: Please add some additional general context regarding the importance of maize in agriculture.

Commented [MOU10R2]: Added

Commented [A11]: This protocol will be published online separately from the TI, so please add additional context. I recommend first adding a sentence about what types of information it would be helpful to know about the maize embryo. Then go into some background about these two techniques and how they will help you gain said information.

Commented [MOU11R2]: Added.

Commented [A12]: Not sure exactly what you mean here.

Commented [MOU12R2]: Since no single-cell or spatial transcriptomic study has worked on maize embryogenesis, we have to optimize the method and protocol to fit the maize embryos.

Commented [A13]: How much is this embryo specific? Would a person be able to reasonably take this protocol and apply it to other tissues using the notes you provided for optimizing to embryo tissue?

Commented [MOU13R2]: In theory, yes, although we didn't test other tissues. The property of some components in plant cells, including cell wall, vacuole and cellular metabolites, may affect the data quality of spatial transcriptomic analyses. The protocol will provide some suggestions in discussion part.

Commented [A14]: I would consider moving a lot of this introduction to the Abstract - you have 250 words. The introduction is meant to be an introduction to the protocol where you tell the reader what they will do, why and how. It should be treated as a roadmap to or a bird's-eye view of the protocol.

Commented [MOU14R2]: Introduction modified to meet the requirement

Commented [A15]: Explain these again here please.

Commented [MOU15R2]: Done

Spatial transcriptomics combines conventional transcriptomics with spatial information, allowing researchers to visualize where specific genes are expressed within sections of whole tissues, organs, and organ systems. Tissue samples are collected and fixed or frozen to preserve both cellular structure and spatial context within the sample. Samples are sliced into thin sections using a microtome or cryostat, and are then mounted onto slides coated with a matrix of oligonucleotide probes that can capture mRNA from the tissue sections. The mRNAs released from these tissue sections are hybridized to probes underlying the tissue sections during the tissue permeabilization process.

The quality of tissue permeabilization is highly influenced by time. Brief permeabilization times may provide insufficient mRNA release from the overlying tissues, resulting in loss of transcriptomic information. In contrast, excess tissue permeabilization may lead to diffusion of the mRNAs, which compromises spatial information. Therefore, spatial transcriptomic protocols require prior optimization of tissue permeabilization times and protocols. During the optimization steps, specially-designed slides are utilized that several mRNA capture areas, thereby enabling testing of multiple tissue sections on a single slide. Tissue sections are then treated with permeabilization reagent for variable time spans, to optimize the protocol. In subsequent RT steps, fluorophore-bound dNTPs are incorporated in the cDNA generated from the permeabilized mRNAs, which enables comparative analyses of the quality of permeabilization at different reaction times. Micrograph samples rendering the brightest and sharpest fluorescent signals enable selection of the optimal protocols in spatial transcriptomic experiments.

As shown in FIGURE 1 and different from the slides used for tissue optimization (TO), each probe on the slides utilized in the actual spatial transcriptomic experiment contains a unique, spatial barcode sequence that is assigned to each specific location on the grid, allowing researchers to retain the spatial context of gene expression data. Also, each probe set on the grid also contains a unique molecular identifier (UMI), which labels every individual captured mRNA molecule. The mRNA released from tissue sections is hybridized to poly-(dT) probes, which bind to the poly(A) tails of processed mRNAs. In the subsequent RT procedure, also distinct from the TO step, dNTPs utilize in the actual spatial RNA-seq experiment do not contain fluorophores. Subsequently, probes are elongated from the poly-(dT) end, following the mRNA template, and are terminated with poly-(dC) tails. A Template Switch Oligo (TSO) has a poly-(rG) sequence at its 3' end, which binds to the poly-(dC) tail and links to the 5' end of the transcript. Using the TSO as a template, the probe is thereby extended via incorporating a sequence complementary to the TSO. The Second Strand Primer, which shares the same 5' sequence as the TSO, then binds to this complementary sequence to initiate second-strand cDNA synthesis. Resulting second strands of the cDNAs are then released from the first strand (extended probe) by denaturation, and collected into a PCR tube for cDNA amplification, library preparation and high throughput sequencing. The resulting data is analyzed to map gene expression back to the original tissue geography, providing insights into spatial patterns of gene activity within tissues and organs.

In this protocol, we provide a tandem streamline of single-cell transcriptomic procedures, followed by an outline for performing spatial transcriptomics. As noted in the protocols, single-cell and spatial transcriptomic analyses each have their distinct advantages and limitations. Whereas single-cell technology allows for high-cellular-resolution profiling of three-dimensional samples, the identification of the tissue-specific location of each cell cluster can be challenging when tissue-specific markers are unavailable in a given plant species. In contrast, spatial transcriptomics offers spatially-resolved transcriptome profiling, but it is limited to two-dimensional sections and suffers from lower cellular resolution and gene-detection sensitivity. Therefore, we recommend combining both approaches to deepen our understanding of the expression landscapes of individual cells and tissues in spatial context.

We optimized these two procedures to fit the unique features of the maize embryo and share our experience determining the developmental stages of the samples, including how they are processed for high throughput single-cell and spatial transcriptomic sequencing, and how to troubleshoot potential experimental issues. This protocol can be readily adapted to investigate embryogenesis of other monocot grasses.

Key words

Single-cell transcriptomic analysis, Spatial transcriptomic analysis, Cryosections, Protoplasting

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Biological Materials and Reagents

Acetone (Millipore Sigma 179124)

Dry Ice

Ethanol, 100% (Millipore Sigma 459844)

Fluorescein diacetate (FDA) (Millipore Sigma 343209)

Glacial acetic acid (Millipore Sigma A6283)

Hydrochloric acid solution, 0.1N (Fisher Scientific SA54-1)

KAPA SYBR FAST qPCR Master Mix (2X) (KAPA Biosystems KK4600)

L-Arginine-HCl (250 mM) with 10 mM MOPS, pH 7.5 (see Recipes)

Leaf 1-staged embryos of maize inbred line B73 (or other line of interest)

(To identify Leaf 1-staged embryo, see METHOD step 1-14)

Methanol, 100% (Millipore Sigma 34860)

MOPS 250 mM, pH 7.5 (See Recipes)

Nuclease-free H₂O (Thermo Fisher Scientific AM9937)

Optimal cutting temperature (OCT) (Tissue-Tek 4583)

Paraplast Plus® (McCormick Scientific 15159-464)

Potassium hydroxide (KOH) Solution, 8M (Millipore Sigma P4494)

Protoplast wash buffer (See Recipes)

Commented [A16]: I'm not sure exactly what you mean here - are these not already characterized?

Commented [MOU16R2]: But we cannot tell the stages from the appearance of maize kernels, and also, we are not able to correspond the kernel age (Day-After-Pollination, DAP) with the specific stages, so before collecting samples, we have to make sure which DAP corresponds to which stage by fast sectioning and microscopy.

Commented [A17]: Please make sure everything listed in the Materials list is explicitly mentioned in the protocol, and vice versa.

Commented [MOU17R2]: Sure.

Commented [A18]: If items in this list are ONLY used within a recipe they should not be listed in the reagents list. Please move any such items and their information to their respective recipes and delete them in this list.

Commented [MOU18R2]: Sure

Commented [A19]: Add to reagents list.

Commented [A20]: Please give additional information on how this stage of embryo is identified and information on this line. You can use a reference if desired but imagine that someone is working with maize for the first time - how would they identify these embryos.

Commented [MOU20R2]: See the method "Maize Embryo Staging" Step 1-14.

Commented [A21]: Can this only be done with B73?

Commented [MOU21R2]: Other lines should be fine.

Protoplasting solution (See Recipes)

Qiagen Buffer EB (Qiagen 19086)

Qubit dsDNA Quantification Assay Kits (Thermo Fisher Scientific Q32850)

RNaseZap (Thermo Fisher Scientific AM9780)

Sodium dodecyl sulfate (SDS) solution, 10% w/v in water (Millipore Sigma 71736)

SPRIselect Reagent Kit (Beckman Coulter B23318)

SSC Buffer 20X (Millipore Sigma S66391L)

SSC 2X with 0.1% w/v SDS

TE Buffer (Thermo Fisher Scientific 12090015)

Tris (1 M, pH 7.0) (Thermo Fisher Scientific AM9850G)

Toluidine Blue (Thermo Fisher Scientific 89640)

Visium Spatial Tissue Optimization Slide & Reagent Kit (10x Genomics PN-1000193)

Visium Spatial Gene Expression Slide & Reagent Kit (10x Genomics PN-1000187)

Universal Mouse Reference RNA (Thermo Fisher Scientific QS0640)

Xylene, 100% (Millipore Sigma, Cat. No. 214736-4L)

Commented [A22]: We probably don't need both the recipe and a catalog number but if you want to keep both that's ok.

Commented [MOU22R2]: The recipe part deleted

Commented [A23]: I assume one of these numbers is incorrect?

Commented [MOU23R2]: fixed

Commented [A24]: I removed micropipettes and tips which are considered to be common items in the lab.

Commented [MOU24R2]: Thanks

Equipment and Supplies

Cell strainers (70 µm; Fisher Scientific 08-771-2)

C-Chip DHC-N01 disposable hemocytometers (Fisher Scientific 22-600-100)

C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad 1851197)

Centrifuge for 50 mL tubes

Compound fluorescent microscope equipped with the red (618 nm excitation filter and a 698 nm emission filter) and green (488 nm excitation filter and a 510 nm emission filter) fluorescent filter setup

Cryomold 10 x 10 x 5mm (Tissue-Tek 62534-10)

Cryostat

Dark-colored paper

Disposable base molds (VWR100501-996)

Embedding ring (VWR 87002-374)

Fine forceps

Fine paintbrushes

Fume hood

Incubation oven

Microcentrifuge

Microtome

Microtome blade (compatible to the corresponding cryostat and microtome)

Millex® PVDF syringe filter (0.22 µm; Millipore Sigma SLGVR33RS)

Tissue embedding machine (Paraffin distributor)

Orbital shaker

Qubit 3.0 (Thermo Fisher Scientific Q33216)

Razor blades (single edge, steel)

Real Time qPCR System

Regular microscope slides (Fisher Scientific 12-550-15)

Sampling vial (VWR 66012-044)

Scalpel

Shaking warm water bath with adjustable temperature

Slide heating platform

Sterile nuclease-free 1.5 mL microcentrifuge tubes

Sterile nuclease-free 15 mL centrifuge tubes

Sterile nuclease-free 50 mL centrifuge tubes

Sterile nuclease-free 96 or 384-well plates compatible with the RT-qPCR system

Sterile nuclease-free PCR 0.2 mL 8-tube strip with cap

Vacuum pump or chamber

Visium Accessory Kit (10x Genomics PN-1000194)

METHOD

Conduct all steps at room temperature unless indicated otherwise.

Maize Embryo Staging

Single-cell and spatial transcriptomics are powerful tools for investigating individual tissues or organs, as well as their relationships, at cellular resolution. According to Takacs et al. (2012), the maize Leaf1 stage embryo is an ideal material for studying embryonic organs since it is the earliest stage when all three types of embryonic lateral organs have emerged. However, determining the exact stages based on

Commented [A25]: Regular is probably relative - please indicate a part number.

Commented [MOU25R2]: Sure

Commented [A26]: Temp?

Commented [MOU26R2]: Variable depending on needs, so the water bath with adjustable temperature is required.

Commented [A27]: A lot of step numbers have changed so please check step numbers throughout.

Commented [MOU27R2]: Got it

Commented [A28]: Okay to add this?

Commented [MOU28R2]: I think it's fine.

maize kernel appearance is challenging, and correlating kernel age (days after pollination, DAP) with specific stages is difficult. Therefore, before sample collection, it is necessary to confirm the DAP corresponding to each stage through rapid sectioning and microscopy. The procedure for identifying Leaf 1 stage maize embryos is outlined in this section. (FIGURE 2)

1. Collect developing kernels from 10 to 15 days after pollination (DAP), every day.
2. Longitudinally cut the kernel on two sides away from the embryo with a single edge razor blade and keep the mid-kernel region containing the embryo (The cutting line is illustrated at FIGURE 2).
3. Embed the mid-kernel region in OCT in the cryomold with the embryo facing top.
4. Freeze the cryomold in dry ice for ~ 15 min.
5. Mount the frozen block on the cryostat.
6. Set the cryostat chamber temperature at -16 °C and set the section thickness to 10 µm.
7. Keep the frozen block and regular slides in the chamber for 30 min prior to sectioning.
8. Cryosection the block and load the medium sections with the embryo shoot apex and coleoptile (as shown in FIGURE 2) on the slide with a fine paintbrush. Usually, we could obtain 3-5 medium section per kernel. (See Troubleshooting)
9. Immediately put a finger on the backside of the section area on the slide for 2-3 seconds to allow the sections to adhere to the slide.
10. Warm up the slide on a heating platform (37 °C) for 1 min, to keep the sections attached on the slide in the subsequent steps. (See Troubleshooting)
11. Immediately place the slide in a Coplin jar with pre-chilled (-20 °C) 100% methanol, and incubate at -20 °C for 30 min.
12. Remove the slide from the Coplin jar and air-dry the slide in a fume hood.
13. Stain the slide in a Coplin jar for 30 seconds with a 0.05% (w/v) Toluidine blue solution with a volume that could fully cover the section area.
14. Wash the slide with the deionized water for 30 seconds.
15. Observe under the compound microscope. The sections of the Leaf1-staged embryo should present the first leaf primordium (Leaf1), SAM, coleoptile (c) and scutellum (sc) as shown in FIGURE 2.
16. Confirm the age (usually 12-13 DAP) corresponding to the Leaf1-staged embryo and collect more embryos of this age for subsequent steps, including preparation for single-cell and spatial transcriptomic analyses.

Sample Preparation for Single-Cell Transcriptomic Analysis

Based on Satterlee and Scanlon (2022), we modified the single-cell transcriptomic procedure for maize Leaf 1 stage embryos (FIGURE 3). The Leaf 1 staged embryos (based on the age determined by step 1-16) are to be used in all the procedure below.

17. Dissect the Leaf 1 staged embryos from freshly collected developing kernels by gently peeling the pericarp covering the embryo using a fine forceps and the embryo will automatically release from the kernel. Then immediately put the embryos into a sampling vial with 10 mL protoplasting solution.
For each batch, to reach sufficient cell yield, collect at least 200 embryos per vial.

Commented [A29]: Elaborate on how you do this

Commented [MOU29R2]: Figure 1 shows the cutting line.

Commented [A30]: What do you cut it with? How do you identify the region containing the embryo? Please keep our audience in mind - explain this as you would to someone doing this for the very first time.

Commented [MOU30R2]: I made it more clear

Commented [A31]: Does the orientation matter?

Commented [MOU31R2]: Yes, I added this information

Commented [A32]: What does this mean? How do you move the sections from the blade to the slide?

How many sections do you normally save per kernel/embryo?

Commented [MOU32R2]: Detail added

Commented [A33]: Please elaborate on these steps in case someone has not done cryosectioning before.

Commented [MOU33R2]: If someone has not done cryosection before, training is required to understand the basic terminologies and procedures.

Commented [A34]: You warm it with the section on it?

Commented [MOU34R2]: Yes. In order to keep the sections attached on the slide in the subsequent steps

Commented [A35]: In what vessel? How much solution? For how long? What temperature?

Commented [MOU35R2]: Added.

Commented [A36]: For how long?

Commented [MOU36R2]: added

Commented [A37]: What are you looking for here? It's also not clear where you proceed from this step.

Commented [MOU37R2]: Added

Commented [A38]: So I assume this is a separate starting point from the cryostat-cut embryos. This is difficult to follow as is but hopefully the text you add to the introduction will help clarify.

Commented [MOU38R2]: Since this part requires leaf1-staged embryos, readers need to know which age (DAP) corresponding to the leaf1-staged embryos (Step 1-16) before collecting the samples.

Commented [A39]: Describe in more detail how you do this.

Commented [MOU39R2]: Added

Commented [A40]: All in the same vial?

Commented [MOU40R2]: Yes

18. To minimize the transcriptome variations caused by different digestion times, complete embryo collection within 1 hour.
Teamwork is required in sample collection.
19. Set the Hg parameter of the vacuum device to 350–400 mm. Put the vial with the lid open in the vacuum chamber and vacuum for 10 min to help the protoplasting solution infiltrate the tissue.
20. Incubate the vial with the embryos in a 28 °C water bath, gently shaking at 90 rpm for 2 hours.
21. During the shaking, prepare the protoplast wash buffer, and keep it on ice.
22. After 2 hours, prepare a 5 mg/mL FDA solution by dissolving 5 mg of FDA in 1 mL acetone. Add 10 µL of the 5 mg/mL FDA solution to the vial of embryos. Gently mix it by tapping the vial and incubate at room temperature in darkness for 5 min.
FDA produces green fluorescence in living protoplasts.
23. Filter the protoplast suspension through a 70 µm cell strainer into a 50 mL centrifuge tube as follows: cut a P1000 micropipette tip and gently transfer the protoplast suspension from the vial onto the cell strainer. Do not pour the protoplast suspension.
24. Wash the cell strainer with 5 mL protoplast wash buffer to collect the remaining protoplasts.
25. Centrifuge the 50 mL centrifuge tube at 250g for 3 min at 4 °C.
26. Carefully remove the supernatant using a P1000 micropipette. Do not pour.
27. Gently pour 15 mL protoplast wash buffer onto the tilted interior wall of the centrifuge tube to resuspend the pellet. Do not straight pour the solution onto the pellet.
28. Repeat Steps 23 to 27 three times, and finally resuspend the pellet with 50 µL of protoplast wash buffer.
29. Take a 10 µL aliquot of the protoplast suspension and load it onto a hemocytometer.
30. Count the number of the living protoplasts under a compound microscope using the GFP filter using the equation at FIGURE 4. Count the number of protoplasts within the areas A, B, C, D and E. The estimated number of protoplasts per µL = $10 \times (\text{dilution factor}) \times (A+B+C+D+E)/5$. The number of living protoplasts should be at least 1,000 – 10,000 per µL of suspension.
 - If the number of the living protoplasts is too numerous to count, dilute the protoplast with the wash buffer to the desired concentration.
 - If the number of the living protoplasts is lower than 1,000, centrifuge the tube at 250g for 3 min at 4 °C, and resuspend the pellet with less than 50 µL of the wash buffer to the desired concentration.
31. Now the living protoplasts are ready for barcoding and library construction. Put the tube with the protoplast suspension on ice and take it to a facility or core that provides the 10x Chromium-based scRNA-seq library preparation service. (See Troubleshooting)

Sample Preparation for Visium Spatial Transcriptomic Analysis

To annotate the identity of the single-cell clusters, we need to know their spatial context. 10X Visium™ is a powerful tool to analyze the spatial transcriptomics. We modified the 10X Visium™ Demonstrated Protocols or User Guide: CG000160, CG000238 and CG000239 to apply to maize Leaf 1 stage embryos.

Sample Collection, Cryosectioning, and Slide Processing

32. Collect developing kernels with the Leaf 1 stage embryo.
33. Follow Step 1-14 for kernel dissection, OCT-embedding, cryosectioning, slide mounting, section fixation as well as section staining using the Visium tissue optimization slides or gene expression slides instead.

Commented [A41]: How do you shake it in the water bath?

Commented [MOU41R2]: There is a type of water bath with a shakable water chamber.

Commented [A42]: Does it actually have to be prepared at this point or can it be prepared before you start the protocol and just chilled at this point?

Commented [MOU42R2]: Never tried it before the protocol started, but I would prepare it at this point.

Commented [A43]: Add to reagents list.

Commented [MOU43R2]: added

Commented [A44]: By pipetting? Turning the vial over? How?

Commented [MOU44R2]: By tapping the vial

Commented [A45]: So the protoplasts go through the cell strainer? What is being strained out?

Commented [MOU45R2]: Yes, the protoplasts will go through the strainer. The large undigested cell aggregate will be strained out.

Commented [A46]: Please add this to the relevant section of the protocol.

Commented [A47]: What do you do if they aren't?

Commented [MOU47R2]: Info added

Commented [A48]: Please provide additional information. Do you take the suspension straight to the facility? Do you have to design any barcodes/sequences or are they universal?

Commented [MOU48R2]: Yes, we take the suspension directly to the facility

Commented [MOU48R3]: The facility will provide the barcoding reagents. The barcodes are universal, so the users do not need to design anything.

Commented [A49]: This doesn't require a core, just access to the equipment, which some groups have in their lab.

Commented [MOU49R2]: Most plant labs do not have the equipment, so I mentioned "facility" or "core" here.

Commented [A50]: Again here, it's unclear how this section relates to the prior sections. Better explanation is needed.

Commented [MOU50R2]: Explanation added.

Commented [A51]: Because these protocols are crucial to the protocol and links often change, we would like to ask you to put all necessary pdfs in a Figshare repository t(... [1]

Commented [MOU51R2]: Good idea.

Commented [A52]: At least one of these is a user gui(... [2]

Commented [MOU52R2]: The term "demonstrated" ... [3]

Commented [A53]: This section is highly redundant w ... [4]

Commented [MOU53R2]: I removed the highly redu ... [5]

34. Observe the slide under the compound microscope and capture the micrograph at 1X objective in bright field. Possible image stitching may be necessary in case the section is too big to be included in one field of view. Software programs such as ImageJ and Photoshop could be used to stitch the micrographs. The micrograph should contain the “fiducial frame” (FIGURE 5), which is a series of dotted markers surrounding the capture area (the area with the sections) for image alignment performed via the software Loupe Browser. Please refer to the corresponding software instructions when analyzing the images.

Tissue Optimization

For tissue optimization, use the Visium Spatial Tissue Optimization (TO) Slide with the sample sections prepared from Steps 32-34. The TO slide contains eight capture areas, four of them for embryo tissue sections, the other four for shoot apex sections or organ of your interest (optional). All reagents or solutions mentioned below are a part of Visium Spatial Tissue Optimization Slide & Reagent Kit.

35. Perform the tissue permeabilization as described in protocol CG000238 (step 1.1 on page 28) with some modifications:
- Add 70 μ L of tissue permeabilization enzyme mix on the maize embryo longitudinal sections for 0 min (negative control), 3 min, 6 min and 12 min at 37°C.
 - We also tested the longitudinal sections of shoot apex of 14 day-after-planting seedlings using the same setup (optional, in order to check if the method also compatible with other type of organs). Users could test any tissues of interest here.
36. Prepare the fluorescent reverse transcription master mix (FRTMM) in a microcentrifuge tube on ice (for the whole slide).
- | | |
|--------------------------------|---------------|
| Nuclease-free H ₂ O | 221.8 μ L |
| RT reagent C | 110.0 μ L |
| Template switch oligo (TSO) | 30.8 μ L |
| Reducing agent B | 8.8 μ L |
| RT enzyme D | 68.6 μ L |
- RT reagent C contains the fluorophore-bound dNTP which could be incorporated in the newly synthesized cDNA that is hybridized with the released RNA after tissue permeabilization. The intensity of fluorescence signal reflects the quality of tissue permeabilization.
37. Remove the tissue permeabilization enzyme from the slide and add 100 μ L 0.1X SSC to each sample capture area.
38. Remove 0.1X SSC from each area by pipetting.
39. Add 50 μ L FRTMM to each sample capture area to initiate reverse transcription. Incubate the reaction at 53 °C for 45 min in the thermocycler.
40. Prepare the tissue removal mix (TRM) in a centrifuge tube at room temperature (for the whole slide)
- | | |
|-----------------------|-------------|
| Tissue removal buffer | 539 μ L |
| Tissue removal enzyme | 77 μ L |
41. Remove the FRTMM from the slide by pipetting and add 100 μ L 0.1X SSC to each sample capture area.
42. Remove 0.1X SSC from each area.
43. Add 70 μ L TRM to each sample capture area and incubate at 56 °C for 1 hour in the thermocycler. Prewarm 45 mL 2X SSC-0.1% SDS in 50 °C water bath for 30 min. Wash the slides with the prewarmed 2X SSC-0.1% SDS, then 45 mL 0.2X SSC, then 45 mL 0.1X SSC by

Commented [A54]: How do you do this and in what c... [6]

Commented [MOU54R2]: Explained

Commented [A55]: Please explain the features of the... [7]

Commented [MOU55R2]: Explained

Commented [A56]: Please check.

Commented [A57]: Does this mean you have 8 embr... [8]

Commented [MOU57R2]: The TO slide contains eigh... [9]

Commented [A58]: This document has 46 pages so... [10]

Commented [MOU58R2]: Added

Commented [A59]: What is this a part of? Please ad... [11]

Commented [MOU59R2]: This enzyme mix is part c... [12]

Commented [A60]: It is not clear how you are able t... [14]

Commented [MOU60R2]: See detail at “CG000238... [13]

Commented [A61]: How and why?

Commented [MOU61R2]: This part is optional. We... [15]

Commented [A62]: In a microcentrifuge tube?

Commented [MOU62R2]: yes

Commented [A63]: Please add whatever kit these re... [17]

Commented [MOU63R2]: I put the sentence prior to... [16]

Commented [A64]: Please give context to this.

Commented [MOU64R2]: added

Commented [A65]: Correct? How long do you incubate it?

Commented [MOU65R2]: Yes, no need to incubate... [18]

Commented [A66]: By pipetting?

Commented [MOU66R2]: Yes

Commented [A67]: Correct?

Commented [MOU67R2]: Yes

Commented [A68]: Is this still done in the thermocycler?

Commented [MOU68R2]: Yes [20]

Commented [A69]: In a centrifuge tube? On ice? Ple... [22]

Commented [MOU69R2]: Yes, in a centrifuge tube... [21]

Commented [A70]: By pipetting?

Commented [MOU70R2]: yes

Commented [A71]: Is this correct? How long do you... [23]

Commented [MOU71R2]: no need to incubate ther... [24]

Commented [A72]: We can't have phrases like this i... [25]

Commented [A73]: Is this still on the slide adapter i... [26]

Commented [MOU73R2]: Yes

Commented [A74]: Are these prewarmed as well? W... [27]

Commented [MOU74R2]: No. 50mL tubes. No.

immersing the slides 15 times in each of the solutions. (All the SSC solutions are in a 50mL conical tube.)

44. Air-dry the slide for 5 min at room temperature.
45. Imaging the slide using a fluorescence compound microscope. Since the dNTP with red-colored fluorophore is incorporated in the cDNA hybridized with the released RNA immobilized on the slide, we use the red fluorescent filter (618 nm excitation and a 698 nm emission) to detect the fluorescence signal from the slides.
46. Interpret the data to decide the appropriate tissue permeabilization time for the Gene Expression Slide as follows (FIGURE 6) (*See Troubleshooting*):
 - Since the sections are removed before imaging, we expect to see nothing in the bright field.
 - The section with optimal permeabilization time (e.g., 6 min in Fig. 5) should show a strong and clear fluorescence signal. In Fig. 5 at 6 min., the outline and details of the tissue are visible.
 - If the time is too short (e.g., 3 min in Fig. 5), a faint and blurry tissue outline will be observed, due to insufficient released RNA; whereas if the time is too long (e.g., 12 min in FIGURE 6), a weak signal without any tissue outline would be observed, due to RNA diffusion.

Spatial Gene Expression Evaluation

This procedure utilizes a Visium Spatial Gene Expression Slide which has 4 capture areas (instead of 8 capture areas on TO slides). And the probe Spatial Gene Expression Slide contains the spatial barcode which is a unique sequence on the probe that is assigned to each spatial location on the slide, allowing researchers to retain the spatial context of gene expression data, whereas the TO slide does not have it. General steps consist of tissue permeabilization, reverse transcription, second strand synthesis, and cDNA amplification. Procedures of reverse transcription and second strand synthesis are demonstrated in FIGURE 1. All reagents or solutions mentioned below are a part of Visium Spatial Gene Expression Slide & Reagent Kit.

47. Permeabilize the tissue on the Visium Spatial Gene Expression Slide with the sample sections prepared from Step 32-34. Perform the same procedure (Step 35) as in the tissue optimization but use "6 min" as the permeabilization time.
48. Remove the tissue permeabilization enzyme and add 100 μ L 0.1X SSC to each sample capture area.
49. Remove the 0.1X SSC from each area by pipetting.
50. Reverse transcription. Perform the same the procedure (Step 36) as in the tissue optimization, except using the "RT reagent" (without fluorophore-bound dNTP) in the reverse transcription master mix.
51. Remove the reverse transcription master mix and add 75 μ L 80 mM KOH to each sample capture area.
52. Incubate the slide 5 min at room temperature.
53. Remove the KOH from each sample capture area by pipetting.
54. Add 100 μ L Qiagen Buffer EB to each sample capture area.
55. Prepare the second strand mix (SSM) on ice (for the whole slide)

Second strand reagent	305.8 μ L
Second strand primer	17.6 μ L
Second strand enzyme	6.6 μ L

Commented [A75]: Please give additional details. I assume you dry the slides after you remove them from the previous step. Anything else besides that before imaging? We also need more details on the system to understand what the signal from the slides is.

Commented [MOU75R2]: Added

Commented [A76]: A great deal more background is needed here - probably belongs in the introduction to the protocol.

Commented [MOU76R2]: Added this in the introduction.

Commented [A77]: Here again I don't think you've ever mentioned how many capture areas there are on this versus the other slide so we will need further introduction on that.

Commented [MOU77R2]: Introduced prior to Step 47.

Commented [A78]: Please use full sentences in the imperative voice. Please refer to specific steps of the protocol.

Do all of these steps take place in the adapter on the thermocycler?

Commented [MOU78R2]: Yes.

Commented [A79]: How long do you incubate?

Commented [MOU79R2]: No need to incubate

Commented [A80]: With a pipette?

Commented [MOU80R2]: Yes

Commented [A81]: Again here, please use full sentences and refer to specific steps.

Commented [MOU81R2]: Fixed

Commented [A82]: By pipetting?

Commented [MOU82R2]: Yes.

Commented [A83]: How long do you leave it on?

Commented [MOU83R2]: No specific incubation time, because it is not critical. If SSM preparation time is 3 min, then leave it for 3 min; if 5 min, then leave it for 5 min.

Commented [A84]: Again please indicate where these reagents came from in the reagents list.

Commented [MOU84R2]: I put "All reagents or solutions mentioned below are a part of Visium Spatial Gene Expression Slide & Reagent Kit." prior to Step 47.

56. Remove the Qiagen Buffer EB from each sample capture area.
57. Add 75 μL SSM to each area and incubate at 65 $^{\circ}\text{C}$ for 15 min.
58. Remove SSM and add 100 μL Qiagen Buffer EB to each sample capture area.
59. Remove Qiagen Buffer EB.
60. Add 35 μL 80 mM KOH to each sample capture area and incubate 10 min at the room temperature to denature and release the second strand from the probe.
61. Add 5 μL Tris (1 M, pH 7.0) to 5 tubes (for 4 samples + 1 negative control) in an 8-tube strip.
62. Using a pipette, transfer 35 μL of the solution containing the second strand cDNA from the slide to the 4 tubes in the 8-tube strips containing 5 μL Tris.
63. Add 35 μL 80 mM KOH to the remaining tube with 5 μL Tris as the negative control.
64. Prepare qPCR mix on ice (for 4 tubes + 1 negative control)

Nuclease-free H_2O	20.4 μL
KAPA SYBR FAST qPCR Master Mix	27.5 μL
cDNA Primers	1.7 μL
65. Add 9 μL of qPCR mix to 5 wells in a 96-well (or 384-well) RT-qPCR plate.
66. Add 1 μL from the samples containing second strand cDNA (from Step 70) to the first 4 tubes containing qPCR mix. For the negative control, add 1 μL of the negative control from Step 71 to the remaining well containing qPCR mix.
67. Run qPCR following the program below:
 - i) 98 $^{\circ}\text{C}$ for 3 min
 - ii) 98 $^{\circ}\text{C}$ for 5 sec
 - iii) 63 $^{\circ}\text{C}$ for 30 sec, signal reading
 - iv) Go to ii for 30 cycles
68. Determine the number of amplification cycles using qPCR. The optimal number of cycles (Cq) should be the cycle corresponding to ~25% of the peak fluorescence value at the exponential phase of the amplification plot (FIGURE 7). (See Troubleshooting)
69. Prepare the cDNA amplification mix on ice in a centrifuge tube (for 4 tubes)

Amp mix	220 μL
cDNA primers	66 μL
70. Add 65 μL of cDNA amplification mix to each of the ~35 μL of solution containing second strand cDNA (from Step 70)
71. Perform cDNA amplification using the following program:
 - i) 98 $^{\circ}\text{C}$ for 3 min
 - ii) 98 $^{\circ}\text{C}$ for 15 sec
 - iii) 63 $^{\circ}\text{C}$ for 20 sec
 - iv) 72 $^{\circ}\text{C}$ for 1 min
 - v) Go to ii for number of cycles determined at Step 76.
 - vi) 72 $^{\circ}\text{C}$ for 1 min
 - vii) 4 $^{\circ}\text{C}$ hold

Store the amplification product at -20 $^{\circ}\text{C}$ for up to 1 week or continue to the subsequent steps.
72. Add 60 μL SPRIselect reagent to each sample to make a total of ~ 100 μL in each tube.
73. Incubate tubes at room temperature for 5 min.

In Steps 74-83, we need a magnet stand with two-sided positions: magnet-High and magnet-Low, for washing and elution, respectively. The magnet stand is included in the Visium Accessory Kit.

74. Take out a magnet stand from Visium Accessory Kit.

Commented [A85]: Add to reagents list.

Commented [MOU85R2]: Added

Commented [A86]: With a pipet?

Commented [MOU86R2]: Yes.

Commented [A87]: Please give details about where these come from and what their composition is.

Commented [MOU87R2]: Mentioned prior to Step 47.

Commented [A88]: What does this mean?

Commented [MOU88R2]: The qPCR thermal cycler collects data at the step iii). Each cycle should generate 1 data point at the step iii) and after 30 cycles there should be 30 data points in total. The built-in analyzing software will connect the 30 points into a curve, reflecting the real-time change of the data.

Commented [A89]: Is Figure 7 an automatic readout from qPCR? If not, please explain how you generated this graph. Also you describe 4 samples plus a control but only 2 samples and a control are shown in the figure.

Commented [MOU89R2]: Figure 7 is an automatic readout from qPCR. I used 2 samples and one control just to describe the concept. It looks much simpler and it is easier to convey the basic concept than too many curves with different colors.

Commented [A90]: More details are needed about these.

Commented [MOU90R2]: Like what type of detail? The two reagents are part of the kit, as I mentioned prior to step 47. Also, I added: prepare the mix "in a centrifuge tube". I don't know if the information is detailed enough or not.

Commented [A91]: Nothing magnetic has been mentioned thus far in this protocol. Greater clarity is needed throughout.

Commented [MOU91R2]: Sure.

75. Place the 8-tube strip onto magnet-high position. Let it sit for 5 min until the solution **clears and** forms a pellet on the tube wall.
76. To wash the pellet, pipet out the supernatant and add 200 μ L 80% ethanol to each tube. Let it sit for 30 sec, and then remove the ethanol.
77. Repeat Step 76 to wash **the pellet again**.
78. Briefly centrifuge the tubes for 3-4 seconds, and place the 8-tube strip onto magnet-low position.
79. Remove all ethanol and air dry the pellets for 2 min.
80. Remove the 8-tube strip from the magnet stand. Add 40.5 μ L Qiagen Buffer EB to each tube to resuspend the **pellet and** incubate for 2 min at room temperature.
81. Place the 8-tube strip back onto the magnet stand in magnet-low position. Let the tubes sit for 2 min until the solution clears.
82. **Transfer 40 μ L of the clear solution containing the purified amplified cDNA to new tubes.**
83. Store the purified amplified cDNA at -20°C for up to 4 weeks or continue to the subsequent step. Send the amplified cDNA to the facility or core that provides the Visium library construction and sequencing services, such as Genomics Facility at Cornell University or Integrated Genomics Operation (IGO) core at Sloan Kettering Institute. The facility will send back the processed data by official Visium analyzing software Space Ranger. Users need to **align** the processed data with the section micrographs based **upon** the spatial barcode using the software Loupe Browser (for detailed instruction, please refer to <https://www.10xgenomics.com/cn/support/software/loupe-browser/latest>). Users could further process the data based on specific needs. For some common analyses, such as spatial clustering and cluster marker gene identification, users could use Seurat (tutorial referring to <https://satijalab.org/seurat/>).

Commented [A92]: Do you remove the ethanol after the second wash?

Commented [MOU92R2]: Yes.

Commented [A93]: I'm confused about what releases the cDNA from the magnets.

Commented [MOU93R2]: Qiagen Buffer EB (step 80) releases the cDNA from the magnets.

Alternative Spatial Gene Expression Sample Preparation Based on the Paraplast-Embedded Sections

*For **plant materials that are difficult to cryosection**, such as **tissues with giant vacuole or thickened cell wall**, an alternative approach is to fix the samples in Farmer's fixative and embed in paraffin blocks. Farmer's fixative is not a crosslinking reagent, and so the RNA molecules targeted in the study are freely released from the tissue onto the Visium slides during the permeabilization step. However, in our experience this method risks RNA quality and yield as compared to cryosection-based methods. Fixation and sectioning procedures are mainly adapted from laser captured microdissection (LCM) sample protocols (Scanlon et al. 2009; Zhang et al. 2018) with modifications to fit the Visium application.*

Commented [A94]: Such as what?

Commented [MOU94R2]: such as tissues with giant vacuole or thickened cell wall

Commented [A95]: Does this need to be freshly prepared or just chilled at this point?

Commented [MOU95R2]: Yes.

Commented [A96]: As above, please provide more details on how you do this and what the size of the mid-kernel region is.

Commented [MOU96R2]: Fixed.

Commented [A97]: What do you cut it with? How do you identify the region containing the embryo? Please keep our audience in mind - explain this as you would to someone doing this for the very first time.

Commented [MOU97R2]: I made it more clear

Commented [A98]: Please write this in the imperative mood: Dehydrate the samples...

Please give more details. Do you remove the fixative from the prior step before doing the dehydration? How much of each of these solutions are you using? Do you do all of these steps in the sampling vial?

84. Freshly prepare Farmer's fixative (ethanol and glacial acetic acid in 3:1 ratio) and chill on ice.
85. **Longitudinally cut the kernel on two sides away from the embryo with a single edge razor blade (the cutting line is illustrated at FIGURE 1) and put the mid-kernel region containing the embryo** into a sampling vial containing 15-20 mL Farmer's fixative). Collect all kernel pieces in a short time frame by limiting the total collection time to 15 min.
86. Set the Hg parameter of the vacuum device to 350-400 mm. Put the vial with the lid open in the vacuum chamber and vacuum for 15 min on ice. Repeat twice more (three times total). Each time after vacuum, remove the old fixative and replace it with a fresh prechill fixative of 20 mL
87. After three changes of fixative, remove the old and replace with the fresh fixative again, cap the vials tightly, and store overnight at 4°C .
88. To dehydrate the sample, remove the old fixative from the prior step and replace it with the following reagents (~20mL each) stepwise **at room temperature**:

100% Farmer's fixative (FF)	1 hour
50% FF, 50% xylene	1.5 hours

- | | |
|--|-----------|
| 100% xylene | 1 hour |
| 100% xylene | 1 hour |
| 100% xylene | 1 hour |
| Paraplast chips (1/10 to 1/5 the volume of the vial) | overnight |
89. Melt paraplast chips in glass beakers 60°C in an oven for infiltration and embedding one night before use.
 90. Transfer the vials into the 60 °C oven, incubate for 1.5 hours.
 91. Add the paraplast chips to 1/2 the volume of the vial to make the paraplast content to 50%, incubate for 1.5 hours in the 60 °C oven.
 92. Pour off the previous paraplast-xylene solution and replace it xylene-paraplast 1:3 mixture to make the paraplast content to 75% and incubate in the 60 °C oven overnight.
 93. Replace the new 100% paraplast in the vial twice a day and keep the vial in the 60 °C oven, for 3 consecutive days.
 94. Install the embedding ring onto the base mold. Transfer the sample to the center of the embedding ring on the base mold. Pour the melted paraplast to fill the embedding ring to make the paraplast sample blocks.
If desired, the blocks can be stored in an airtight bag in 4°C for future use.
 95. Bring the paraffin blocks containing embedded samples to room temperature. Use a blade to cut a piece of the block. Trim the tissue block and mount onto plastic blocks for sectioning. Further trim the tissue block to obtain trapezoid-shaped sections, better leaving at least 1 mm perimeter of paraplast around the tissue.
 96. Mount the block onto the block holder of the microtome by positioning the longer of the parallel edges of the trapezoid at the bottom
 97. Obtain 10 µm thick sections as ribbons using a rotary microtome and transfer the ribbon on to a dark-colored paper using two fine paintbrushes (each paintbrush holds one end of the ribbon). Cut the ribbon and trim the sections to less than 6 mm × 6 mm with a razor blade.
 98. Pipet a drop of nuclease-free water at the capture areas on the Visium Spatial Tissue Optimization slide or the Visium Spatial Gene Expression slide.
 99. Use a fine paintbrush to move a section and mount it on the water and incubate the slide on the heating platform at 40 °C for 10 min.
 100. Gently pipet out the remaining water from under the sections and make sure the sample does not cover the fiducial frame.
 101. Dry the slide on the 40 °C heating platform overnight.
 102. Deparaffinize the slides by immersion in the following reagents: 100% Xylene (two times, 10 min each), then 95% ethanol (two times, 10 min each), then airdry in the fume hood. Perform all these in 50mL conical tubes with 45 mL corresponding solutions.
 103. Warm up the slide on the Visium Thermocycler Adaptor (pre-warmed on a thermocycler for at least 20 min at 37 °C) for 1 min.
 104. Immediately put in a Coplin jar with overnight pre-chilled (-20 °C) 100% methanol, and incubate in -20 °C for 30 min.
 105. Airdry the slide in the fume hood.
 106. The slide is ready for staining (Step 12-14), imaging (Step 34) and subsequent experiments of Visium tissue optimization (Step 35-46) or gene expression (Step 47-83).

Troubleshooting

Problem (Step 8): Tissues break during cryosectioning.

Commented [A99]: Are these melted at this point?

Commented [MOU99R2]: No, it will gradually melt but it's fine if there is still some residue left at the bottom of the vial after overnight incubation.

Commented [A100]: I'm not following what is happening here, please clarify.

Commented [MOU100R2]: Step 89, Paraplast chips should be melted one night before use to make sure they are completely melted. The purpose of doing this is to provide sufficient melted paraplast for subsequent steps. Step 90, transfer the vial into 60C oven for 1.5h in order to complete melt the remaining paraplast chips.

Commented [A101]: Paraplast?

Commented [MOU101R2]: Yes, fixed.

Commented [A102]: This is all very difficult to follow; please clarify.

Commented [MOU102R2]: The purpose of all these steps is to gradually increase the content of the paraplast: from 1/10-1/5 (or 10%-20% at Step 88) → 1/2 (or 50% at Step 91) → 3/4 (or 75% at Step 92) to 100% (at Step 93). We made 6 changes (twice a day X 3 days) of 100% paraplast in order to make sure the sample is filled with absolute paraplast and no xylene left in the sample.

Commented [A103]: Please elaborate.

Commented [MOU103R2]: Done

Commented [A104]: What size?

Commented [MOU104R2]: better leaving at least 1 mm perimeter of paraplast around the tissue.

Commented [A105]: This doesn't really make sense, especially with no information about the paper being used. Please add the paper to the equipment list.

Commented [MOU105R2]: Added

Commented [A106]: At 40C again?

Commented [MOU106R2]: Yes.

Commented [A107]: Please write this in the active, imperative mood. Please explain what type of container is used to do these steps and how much of each reagent is used.

Commented [MOU107R2]: Fixed

Commented [A108]: You warm it up and then put it straight into cold methanol? Just checking.

Commented [MOU108R2]: Yes. Warm it up for better attachment, and cool it down in -20C methanol for re... [28]

Commented [A109]: Please indicate specifically whe... [29]

Commented [MOU109R2]: Added

Commented [A110]: Please update these Step num... [30]

Commented [MOU110R2]: Fixed and added

Solution: Optimize the chamber temperature, and keep the sample blocks in the chamber for at least 30 min to equilibrate the blocks to the optimal temperature in the chamber; optimize the freezing condition or try other cryo-embedding medium, such as NEG50, SubXero and TFM, etc. Alternatively, try the paraplast-embedded section-based method.

Problem (Step 10): Sections become detached from the slides.

Solution: When mounting the section on the slide, place a finger on the backside of the capture area on the slide for a few seconds to allow the section to adhere to the slide. Also, before methanol fixation, warm up the slide for 1 min at 37°C on the Visium Thermocycler Adaptor (the thermocycler should be pre-warmed at 37°C for at least 20 min).

Problem (Step 31): Protoplast yield is low.

Solution: Increase the amount of starting materials; optimize the digesting enzyme concentration and digesting time; gently transfer the protoplasts and gently handle the tubes with the protoplasts; optimize the condition of digesting enzyme solution and the wash buffer (such as pH, osmolarity, etc.)

Problem (Step 31): Protoplasts are aggregated.

Solution: Filter the protoplast suspension for one or two more times; dilute the protoplast before mounting on the hemocytometer.

Problem (Step 46): No fluorescent signal is observed on the Tissue Optimization slides.

Solution: Mount 1-2 µL of positive control (purified RNA) on the Tissue Optimization slide and check if it has any signal; increase the tissue permeabilization time; before permeabilization, treat the sections with 2% (w/v) PVP40 solution (to remove secondary metabolites that limit the enzyme activities required in the procedure) and incubate in 37°C for 15 min (or optimized time).

Problem (Step 46): The fluorescent signal is diffused on the Tissue Optimization slides.

Solution: Shorten the permeabilization time.

Problem (Step 68): Abnormal cDNA trace is obtained with flat shapes or multiple peaks.

Solution: Optimize the amount of Tris (1M, pH 7.0) added at Step 72.

DISCUSSION

Advancements in transcriptomic technologies have opened new avenues for developmental biology research, where single-cell and spatial transcriptomic techniques provide powerful toolkits to unravel the intricacies of cell and tissue-specific gene expression at unprecedented resolution. However, these techniques have yet to be widely applied to studies of plant organ development. In this protocol, we used maize as a model system and modified conventional procedures to better suit the intricacies of plant cells and tissues.

For example, cryosection for plant tissues is challenging, mainly due to the cell wall and vacuole. Rigid plant cell walls comprise cellulose, lignin, and other polysaccharides that are rendered tough and brittle when frozen, making it difficult to generate clean tissue sections without physically damaging the tissue. Water-filled vacuoles form ice crystals, which can expand and rupture cells or distort tissue structure. To overcome those limitations, we optimized the conditions for rapid freezing and cryosectioning. We tested

Commented [A111]: If this is the only place this is used it should not be in the reagents list.

Also please indicate the purpose of this reagent.

Commented [MOU111R2]: Fixed.

Commented [A112]: Please add a discussion section.

isopentane and liquid nitrogen baths followed by dry ice (as described in 10X demonstrated protocol *CG000240*), versus dry ice only to freeze the samples. We found that the dry-ice-only protocol gave better section quality, perhaps because the former method rendered the tissue too rigid to section smoothly. In addition, we tested different cryostat chamber temperatures and found that -16°C is optimal for maize embryo tissues. If the temperature is too low, the tissue blocks become rigid and may cause tissue tearing; if the tissue temperature is too high, the block becomes softer and difficult to cut, leading to distorted sections with unclear morphology.

This protocol may be applied to other plant tissues or organs, with possible modifications according to cellular structures. If the tissue or organ contains large vacuoles, such as mature leaf cells or immature starchy endosperm cells, we recommend applying cryoprotectant solutions (such as sucrose) before freezing. This can help reduce formation of ice crystals by removing excess water from the tissues. If the tissue or organ contains thickened cell walls such as collenchyma or the basal endosperm transfer layer, polysaccharide degrading enzymes such as cellulase, xylanase, and mannanase, etc. can be applied to partially digest the cell wall (Giacomello et al., 2017). Furthermore, we offer a paraplast-embedding-based approach as an alternative sample preparation method for spatial transcriptomics, in cases where cryosectioning is not feasible. Nevertheless, due to the diverse morphology and structure of plant cells and tissues, researchers may need to optimize conditions on a case-by-case basis.

Single-cell transcriptomic assays enable the collection of high-throughput sequencing data, revealing the expression levels of thousands of genes in individual cells. By clustering cells based on their differential gene expression patterns, these data can help identify distinct cell types, subtypes, or states within a tissue or organ (e.g., Satterlee et al. 2020). Analysis pipelines can be used to infer cluster-specific marker genes, co-expression networks, and gene regulatory interaction - key elements toward understanding cellular function and inter-cluster relationships (e.g., Wu et al. 2024).

Spatial transcriptomic analyses provide high-throughput sequencing data that is mapped to defined spatial coordinates within tissue sections. By correlating gene expression with specific spatial domains in the sample, this technique can identify locations of different cell or tissue clusters while preserving their spatial relationships. These analyses enable inferences of spatial cluster-specific marker genes and the identification of co-expression networks regulating the corresponding clusters (e.g., Wu et al. 2024).

However, single-cell transcriptomic analysis can be limited in model systems where tissue/organ marker genes are not previously identified, making it challenging to infer cell type identities of all clusters. To address this limitation, we suggest combining single-cell RNAseq and spatial transcriptomic data by analyzing the overlap of cluster-specific genes between the two approaches. The identity of single-cell clusters can then be inferred, based upon their overlap with spatial clusters sharing corresponding cluster-specific marker genes. For instance, in Fig. 2h of Wu et al. (2024), single-cell cluster #0 (sc_cluster 0) shows strong overlap with spatial clusters V0, V3, and V4 corresponding to the scutellum, suggesting that sc_cluster 0 likely represents a scutellum cluster.

In summary, we present a streamlined protocol that integrates single-cell transcriptomics with spatial transcriptomics. Each technique offers distinct advantages and faces specific limitations. Combining these complementary approaches enables a more comprehensive understanding of gene expression patterns in individual cells within spatial contexts.

RECIPES

L-Arginine-HCl (250 mM) with 10 mM MOPS, pH 7.5

To prepare 50 mL solution add:

L-Arginine-HCl (Millipore Sigma 181003)	2.63 g
3-(N-morpholino) propane sulfonic acid (MOPS) (Millipore Sigma 69947)	0.105 g
Add nuclease-free water to	45 mL
Adjust pH to 7.5 with 10N NaOH	
Add nuclease-free water up to 50 mL.	

(Better prepared fresh, or store in 4 °C for no longer than a month)

Commented [A113]: With what?

Commented [MOU113R2]: with 10N NaOH

Commented [A114]: Is this freshly prepared each time? If not indicate how it is stored and for how long.

Commented [MOU114R2]: Better prepared fresh, or store in 4C for no longer than a month

MOPS 250 mM, pH 7.5

To prepare 50 mL solution add:

MOPS	2.616 g
------	---------

Add nuclease-free water up to 45 mL.
Adjust pH to 7.5 with 10N NaOH
Add nuclease-free water up to 50 mL.
(Better prepared fresh, or store in 4 °C for no longer than a month)

Commented [A115]: Prepared fresh? If not please provide storage conditions and length.

Commented [MOU115R2]: Better prepared fresh, or store in 4C for no longer than a month

Protoplasting solution

To prepare 10 mL solution add:

Cellulase RS (Research Products International C32400-10.0)	150 mg
Cellulase R10 (Research Products International C322005.0)	150 mg
Macerozyme R10 (Research Products International M22010-5.0)	100 mg
Hemicellulase (Millipore Sigma H2125-150KU)	100 mg
Mannitol (Millipore Sigma M4125)	1.184 g
MOPS (250 mM; pH 7.5)	400 µL

Incubate the mixture of the above components at 55 °C for 10 min.
Cool to room temperature and then add:

L-Arginine-HCl (250 mM; pH 7.5)	400 µL
β-mercaptoethanol (Millipore Sigma 444203)	3.5 µL
Bovine serum albumin (BSA) (Millipore Sigma A8022)(5% w/v)	200 µL
CaCl ₂ (Millipore Sigma C4901) (1M)	10 µL

Freshly prepare.

Protoplast wash buffer

To prepare a 100 mL solution add:

Mannitol	11.84g
MOPS (250 mM; pH 7.5)	4.0 g
L-Arginine-HCl	4.0 g

Commented [A116]: Is this a mistake? I thought the pH 7.5 referred to the 250 mM solution.

Commented [MOU116R2]: 250 mM; pH 7.5

Add nuclease-free water to a final volume of 100 mL

Freshly prepare and keep on ice.

COMPETING INTEREST STATEMENT

The authors report no competing interests

AUTHOR CONTRIBUTIONS

H. Wu wrote the manuscript and generated all the Figures and sample data. M.J. Scanlon edited the document.

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Figure legends

FIGURE 1 Procedure for reverse transcription and second strand cDNA synthesis

Adapted from Visium Demonstrated Protocol CG000239. Using the captured transcript as the template, the probe is elongated via reverse transcription, and forms a cDNA strand with poly-dC at the 3' end. The Template Switch Oligo (TSO) has poly-rG at the 3' end, which can bind to the poly-dC, and connect with the 5' end of the transcript. Then, using the TSO as the template, the cDNA-containing probe is extended to have sequence complementary to the TSO sequence. The Second Strand Primer, which shares the 5' sequence with the TSO, binds to the complementary TSO sequence to start the second strand cDNA synthesis. The second strand contains TSO, transcript sequence, poly-dT, unique molecular identifier (UMI), spatial barcode (a unique sequence assigned to each spatial location) and Read 1 (constitutive sequence of the probe, not unique or specific). After the denaturation, the second strand is released from the first strand (the extended probe immobilized on the slide) and collected into a PCR tube.

FIGURE 2 General procedures for maize embryo staging

- Cut the developing kernel longitudinally the following the black line.
- Remove the two sides of the kernel and keep the mid-kernel region with the embryo.

Commented [A117]: I assume this has to be freshly prepared?

Commented [MOU117R2]: yes

Commented [A118]: These sections are both required. Please see our instructions for further information.

Commented [A119]: Please make sure the font on all figures is Helvetica

Commented [MOU119R2]: Yes

Commented [A120]: This is very useful but this should all be explained in the text as well.

Commented [MOU120R2]: Added it in the text

Commented [MOU121]: This part has been moved to FIGURE1 as it was mentioned first in the protocol introduction.

Commented [A122]: Define

Commented [MOU122R2]: Done

Commented [A123]: Provide context.

Commented [MOU123R2]: Added

Commented [A124]: Please provide text explaining the rest of the figure.

Define OCT in the legend.

Commented [MOU124R2]: Added.

- C. Embed the mid-kernel region with the Optimal Cutting Temperature (OCT) reagent in a cryo-mold.
- D. Section using a cryostat.
- E. Mount the sections on to a regular slide
- F. Fix the sections with the -20°C chilled methanol in a Coplin jar
- G. Stain with Toluidine Blue and observe the micrograph. The micrograph shows the longitudinal mid-section of a Leaf1 staged maize embryo, where sc=scutellum, c=coleoptile, Leaf1=first foliar leaf, SAM=shoot apical meristem, Scale bar=100 μm .

FIGURE 3 Sample preparation procedures for single-cell transcriptomic analysis

- A. Collect the Leaf1 staged developing kernels, and release the embryo by gently peeling the pericarp covering the embryo.
- B. Transfer the embryos into a vial containing 10 mL of the protoplast solution.
- C. Vacuum the embryos with the vial lid open.
- D. Transfer the vial into a 28°C water bath shaker and incubate for 2 hours.
- E. Filter the protoplast suspension through a 70 μm cell strainer.
- F. Centrifuge the suspension with 250xg for 3 min in 4°C .
- G. Remove the supernatant and resuspend the pellet with 15 mL of the wash buffer, followed by centrifugation. Repeat these steps for 3 times.
- H. Resuspend the pellet with 50 μL of the wash buffer.
- I. Take a 10 μL aliquot of the protoplast suspension and load it onto a hemocytometer. Count the number of the living protoplasts under a compound microscope

FIGURE 4 Estimation of protoplast number using a hemocytometer

Count the number of protoplasts within the magenta-highlighted areas A, B, C, D and E. The estimated number of protoplasts per $\mu\text{L} = 10 \times (\text{dilution factor}) \times (A+B+C+D+E)/5$

FIGURE 5 Captured micrograph after staining

The captured image was stitched by Adobe Photoshop CS6. The fiducial frame was aligned using Loupe Browser 8.0. The stitched micrograph shows a Toluidine blue-stained mid-longitudinal section of a developing kernel with the Leaf1 staged embryo.

FIGURE 6 Tissue optimization of the maize embryo and shoot apex

The maize Leaf 1 stage embryo (upper low) and 14 day after planting shoot apex (lower low) sections are treated with tissue permeabilization enzyme mix for 0 min (negative control), 3 min, 6 min, and 12 min. The result shows that 6 min is the optimal permeabilization time for both the embryo and the shoot apex. Scale bar = 200 μm . The white dotted lines are showing outline of organs.

FIGURE 7 Example qPCR Amplification Plots

This experiment aims to determine the number of amplification cycles. The purified second strand cDNA was used as the template for the amplification. The optimal number of cycles (Cq) should be the cycle corresponding to $\sim 25\%$ of the peak fluorescence value at the exponential phase of the amplification plot. As the example shows, blue, red and green curve represent the negative control, Sample 1 and 2, respectively. The purple dash line marks the 25% of the peak fluorescence value, so the corresponding Cq value (marked by the arrows) of the Sample 1 and 2 are 17 and 17.5 (rounded to 18), respectively.

Commented [A125]: Indicate what leaf1 means and indicate here that this is a maize embryo and how it's been treated.

Commented [MOU125R2]: Added.

Commented [A126]: How do you shake it in the water bath?

Commented [MOU126R2]: There is a type of water bath with a shakable water chamber.

Commented [A127]: Please add this to the relevant section of the protocol.

Commented [MOU127R2]: Added.

Commented [A128]: Please explain what we are seeing in the figure.

Commented [MOU128R2]: Sure.

Commented [A129]: It's not really clear why you are testing this here because you never mention this age plant/section again throughout the protocol.

Also, you have these rows labeled embryo 10x and embryo 5x. Which is the shoot apex?

Finally, please indicate in the legend what the white dotted lines are showing.

Commented [MOU129R2]: In the protocol, I added the information about the shoot apex, which is: "this part is optional. We did this to check if the method also compatible with other type of organs. You could test any tissues of interest here".

The "embryo 5x." actually should be "shoot apex 5x". It was a type, and has been fixed.

The white dotted lines are showing outline of organs.

Commented [A130]: Please give more details about the experiment here.

Commented [MOU130R2]: Provided.

Page 17: [1] Commented [A51]

Author

Because these protocols are crucial to the protocol and links often change, we would like to ask you to put all necessary pdfs in a Figshare repository that will allow access to these files. We can also add a note here that indicates to check the company website for updates.

Page 17: [2] Commented [A52]

Author

At least one of these is a user guide instead of a demonstrated protocol; please use accurate terminology.

Page 17: [3] Commented [MOU52R2]

Microsoft Office User

10/12/24 9:05:00 PM

The term "demonstrated protocol" and "user guide" are interchangeable.

Page 17: [4] Commented [A53]

Author

This section is highly redundant with the first section of this protocol. I think we could probably streamline them together with appropriate notation.

Page 17: [5] Commented [MOU53R2]

Microsoft Office User

10/12/24 9:33:00 PM

I removed the highly redundant part and pointed out the differences from the prior similar steps ("using the Visium tissue optimization slides or gene expression slides instead.")

Page 18: [6] Commented [A54]

Author

How do you do this and in what case would you do this?

Page 18: [7] Commented [A55]

Author

Please explain the features of the slide - what is the fiducial frame and what is the capture area?

Page 18: [8] Commented [A57]

Author

Does this mean you have 8 embryos on this slide? Please explain.

Page 18: [9] Commented [MOU57R2]

Microsoft Office User

10/16/24 1:33:00 PM

The TO slide contains eight capture areas, four of them for embryo tissue sections, the other four for shoot apex sections. Four embryo or shoot apex tissue sections could be obtained from a same embryo or shoot apex sample.

Page 18: [10] Commented [A58]

Author

This document has 46 pages so it would be helpful to refer to a specific step here.

Page 18: [11] Commented [A59]

Author

What is this a part of? Please add everything mentioned in the protocol to the reagents list.

Page 18: [12] Commented [MOU59R2]

Microsoft Office User

10/16/24 1:55:00 PM

This enzyme mix is part of Visium Spatial Tissue Optimization Slide & Reagent Kit which has already been listed.

Page 18: [13] Commented [MOU60R2] Microsoft Office User 10/16/24 1:58:00 PM

See detail at "CG000238 (step 1.1 on page 28)"

Page 18: [14] Commented [A60] Author

It is not clear how you are able to do this time course on a single slide.

Page 18: [15] Commented [MOU61R2] Microsoft Office User 10/16/24 2:02:00 PM

This part is optional. We did this to check if the method also compatible with other type of organs. You could test any tissues of interest here, just follow the same sample collection, cryosectioning and slide processing steps (as step 33)

Page 18: [16] Commented [MOU63R2] Microsoft Office User 10/16/24 2:09:00 PM

I put the sentence prior to step 35: "All reagents or solutions mentioned below are a part of Visium Spatial Tissue Optimization Slide & Reagent Kit."

Page 18: [17] Commented [A63] Author

Please add whatever kit these reagents are part of to the reagent list.

Additional explanation is needed regarding the purpose of the various components of this mix (either here or in the introduction).

Page 18: [18] Commented [MOU65R2] Microsoft Office User 10/16/24 2:12:00 PM

Yes, no need to incubate them. Add and then remove.

Page 18: [19] Commented [A67] Author

Correct?

Page 18: [20] Commented [MOU68R2] Microsoft Office User 10/16/24 4:01:00 PM

Yes

Page 18: [21] Commented [MOU69R2] Microsoft Office User 10/16/24 4:11:00 PM

Yes, in a centrifuge tube, at room temperature. All components are from the kit (I mentioned it above).

Page 18: [22] Commented [A69] Author

In a centrifuge tube? On ice? Please indicate where these components come from.

Page 18: [23] Commented [A71]

Author

Is this correct? How long do you incubate before removing?

Page 18: [24] Commented [MOU71R2]

Microsoft Office User

10/16/24 4:10:00 PM

no need to incubate them. Add and then remove.

Page 18: [25] Commented [A72]

Author

We can't have phrases like this in the steps. This should be converted into instructions. You will also need to mention in the protocol introduction that tissue removal is a step.

Page 18: [26] Commented [A73]

Author

Is this still on the slide adapter in a thermocycler?

Page 18: [27] Commented [A74]

Author

Are these prewarmed as well? What type of container are these in? Do you keep the 2x SSC-0.1% SDS in the waterbath while you are dipping?

Page 22: [28] Commented [MOU108R2]

Microsoft Office User

10/16/24 11:03:00 PM

Yes. Warm it up for better attachment, and cool it down in -20C methanol for re-fixation.

Page 22: [29] Commented [A109]

Author

Please indicate specifically where a user would proceed in the above sections from here.

Page 22: [30] Commented [A110]

Author

Please update these Step numbers and under the relevant Step write "See Troubleshooting."