Chapter Title:

Optimizing Xenium *In Situ* for Spatially Resolved Gene Expression Profiling in *Medicago truncatula* Roots and Nodules

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Abstract

Elucidating the intricacies of nodule organogenesis at a spatial-temporal level is pivotal for advancing our grasp of developmental biology. This knowledge paves the way for precise agricultural modifications, enabling the incorporation of beneficial traits into crops while avoiding adverse effects. However, exploring the elaborate gene regulatory networks during plant organogenesis within the context of native spatial tissues poses significant challenges. Spatial transcriptomics technologies developed for animal systems are often not directly transferable to plant tissues due to the heterogeneous nature of plant cell walls, high autofluorescence, and large vacuoles in plant cells. To address these challenges, we present an optimized protocol for applying the Xenium *in situ* platform to formalin-fixed paraffin-embedded (FFPE) sections of plant tissues, including *Medicago truncatula* roots and nodules. Key technical adaptations include customized tissue preparation, optimized section thickness, hybridization conditions, post-Xenium staining, imaging, and downstream image analysis, all tailored specifically for plant samples. To mitigate autofluorescence and enhance detection sensitivity, we employed strategic codeword selection during probe design. Furthermore, we developed a modular probe design approach combining a 380-gene standalone panel with a 100-gene add-on panel. This design allows flexibility for addressing diverse research questions and includes orthologous gene sequences from two Medicago ecotypes, ensuring compatibility for downstream functional validation using mutant lines available in both genetic backgrounds. We validated the protocol across nodules at multiple developmental stages—1, 2, 3, 4, 14, and 28 days post-inoculation (DPI)—using both the 50-gene panel targeting mature nodule cell identity and the extended 480-gene panel, which includes markers across different cell types and developmental stages, as well as genes of interest identified from prior single-cell and bulk RNA-seq analyses. This optimized workflow provides a reproducible and scalable method for

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high-resolution spatial transcriptomics in plant tissues and establishes a robust foundation for adaptation to other plant species and developmental systems.

Key Words

Medicago truncatula, Xenium *in situ*, spatial transcriptomics, nodulation, gene expression, root nodules, nitrogen fixation, plant tissue sectioning

2 Introduction

Certain plants have developed extraordinary adaptive traits that allow them to thrive under conditions such as nutrient deficiency, notably through the ability to fix atmospheric nitrogen (N_2) via symbiosis with bacteria in root nodules. These capabilities present a substantial opportunity to boost agricultural productivity *(1)*. However, unraveling the genetic foundations conferring these adaptive advantages has been challenging through traditional genetics or bulk RNA sequencing methods. This difficulty arises because the emergence of such traits often involves altering the regulatory mechanisms of genes that are otherwise conserved, leading to novel spatial or temporal gene expression patterns—a phenomenon observed across both the plant and animal kingdoms. For example, genes that typically play a role in lateral root development have been repurposed in some plants to facilitate the formation of nitrogen-fixing nodules *(2)*.

The potential agricultural benefits of these adaptive traits are immense~~,~~ but identifying the precise genetic mechanisms that underline their development poses a formidable challenge. These traits often result from evolutionary changes that modify the developmental fate of particular cell types. For instance, cells in the cortex, endodermis, and pericycle can be reprogrammed from their original role in lateral root formation to create nitrogen-fixing nodules. Pinpointing the transcriptional differences in cell lineage that lead to such divergent developmental outcomes is crucial in unraveling how distinct organ structures and functionalities emerge. Traditional approaches to studying cell lineage regulation in plant development have primarily focused on analyzing individual genes, often employing targeted mutations. However, these methods, including bulk RNA sequencing, lack the resolution to observe changes in gene expression patterns at the cellular level. Creating a detailed atlas covering the organogenesis of Medicago nodules, which form a mutualistic symbiosis with nitrogen-fixing rhizobia, is essential for understanding the complex interplay of cell types

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within. Grasping the development and differentiation of various cell lineages into distinct cell types and functions is a pivotal initial step in deciphering the gene regulatory networks that drive the formation of this specialized organ.

Single-cell transcriptomics has revolutionized plant research by allowing scientists to group cells into populations with similar molecular characteristics, facilitating a deeper understanding of cell types and their conditions *(3)*. As these technologies evolve, offering higher throughput and sensitivity, they enable cells to be categorized with unprecedented detail. However, this advancement also introduces challenges, particularly when we encounter cell populations that fall outside our existing knowledge of plant cell histology and physiology. As discussed in previous studies *(4)*, examining the molecular identity of these cell populations and their spatial arrangement within plant tissues is essential to fully comprehend their roles and interactions. One of the go-to methods for mapping the spatial distribution of cell population markers discovered through single-cell transcriptomics is using transgenic reporter lines, which express fluorescent proteins driven by the predicted promoters of target genes. Although effective for showing single gene activity, this method struggles with complex tissues because cell identity often depends on multiple genes. Additionally, generating transgenic plants is time-consuming, and the artificial expression may not reflect true gene activity due to missing genomic context, like enhancer-promoter interactions. While *in situ* hybridization offers a partial solution by addressing some limitations, it lacks the ability to analyze many genes at once due to low multiplexing capabilities. Hence, comprehensive spatial gene expression analysis, crucial for understanding cell functions and interactions, requires examining a broad spectrum of genes at the single-cell level.

The advent of spatial transcriptomics is set to overcome these obstacles by providing detailed insights into both the molecular composition and the physical locations of cells within complex tissues *(5, 6)*. Spatial transcriptomics techniques, such as spatially barcoded arrays *(7–9)* and multiplexed fluorescence *in situ* hybridization *(4, 10)*, enable the study of extensive gene sets along with their spatial contexts. This advancement is particularly significant in plant research, opening new possibilities for exploring the complex interplay of plant cells and tissues *(4, 7–10)*. However, the task of investigating complex gene regulatory networks during plant organogenesis, within their native spatial contexts, remains challenging. Adapting spatial transcriptomics methods from animal to plant tissues is especially difficult due to the unique structure of plant cell walls and the presence of large vacuoles within plant cells.

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- it depends on protoplasting, very limiting and introduces biases
- cell type identity is context dependent; the identity of a given cell is also defined by the identity of neighbour cells and the whole tissue in which it is situated. Data indicate that cell identity is affected within hours of protoplasting. Again, this is likely to introduce biases not present in the spacial-transcriptomics.

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In response to these difficulties, we present a new protocol using Xenium *in situ*, a highly multiplexed fluorescence *in situ* sequencing technique, optimized for plant tissues. This method enables subcellular precision and high sensitivity, even in Formalin-Fixed Paraffin-Embedded (FFPE) sections, providing a detailed spatiotemporal view of gene expression. By analyzing 50 genes, including cell-type-specific markers, across different stages of nodule development in *Medicago truncatula*, this protocol delineates primary tissue layers and cell types, offering new insights into the spatial organization of gene activity.

Xenium *in situ* allows for the spatial resolution of gene expression patterns previously identified in single-cell RNA sequencing datasets, enhancing our understanding of complex tissue dynamics. This breakthrough technique surpasses the limitations of traditional approaches, enabling the study of plant organogenesis at an unprecedented level of detail. It not only provides a powerful tool for developmental biology research but also opens new possibilities for translating these findings into agricultural innovations that enhance crop resilience and yield.

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3 Materials

2.1 Probe Design

2.1.1 Software and Resources

- 10x Genomics Xenium Designer
- *Medicago truncatula* genome annotation
- Gene expression datasets (e.g., bulk RNA-seq, scRNA-seq)
- List of 50-480 target genes (transcript IDs and gene names)

2.2 Tissue Fixation and Paraffin Block Preparation

2.2.1 Reagents and Solutions

- 1× **PBS Buffer:** 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl (pH 7.2–7.4)
- Fixative (4% paraformaldehyde)
- Glutaraldehyde (GA, 25%)
- Ethanol (10%, 30%, 50%, 70%, 90%, 100%)
- Histo-Clear II
- Paraplast Xtra (melted, 58–60 °C)
- MQ water (autoclaved and de-gassed)

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2.2.2 Equipment and Tools

- Glass vials
- Vacuum desiccator
- Embedding molds
- Heating plate (60 °C)
- Dissecting microscope
- Ice or cooling plate
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2.3 Paraffin Sectioning and Section Slide Preparation

2.3.1 Reagents and Solutions

2.3.2 Equipment and Tools

- Microtome with disposable knives
- Heating plate (42°C)

2.4 Probe Hybridization and Processing Xenium Slides

2.4.1 Reagents and Solutions

2.4.2 Equipment and Tools

- Xenium analyser

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3 Methods

3.1 Probe Design for Xenium Panel

3.1.1 Target Gene Selection

1. Select genes based on experimental questions (e.g., nodulation).
2. Use transcriptomic data to identify marker or enriched genes.

3.1.2 Panel Submission and Review

1. Format the target list in a 10x template (CSV).
2. Submit to Xenium Design Portal.
3. Review QC feedback and adjust the target list if needed.
4. Finalize design and order panel (allow ~3–4 weeks for delivery).

3.2 Tissue Fixation and Paraffin Block Preparation

3.2.1 Buffer Preparation

Day1: Preparing Fixative Solution

1. To prepare 30mL fixative solutions, add **80 µL 1N NaOH** to **24 mL of water** in a glass beaker. This step helps adjust the pH for dissolving paraformaldehyde (PFA).
2. Warm the solution slightly in a microwave. Heat for **10 seconds**, repeating 4 times, and mix well after each round.
3. Add **1.2 g PFA** to the warm solution. Stir continuously until the PFA fully dissolves. The solution may appear cloudy initially but will clear upon complete dissolution.
4. Cool the PFA solution on ice until it reaches 4°C.
5. Add **300 µL glutaraldehyde (25%)** and stir gently.
6. Finally, add **6 mL of 5× PBS** to the mixture to achieve the desired buffer strength.
7. Dispense the fixative into clean glass vials and keep them on ice to maintain freshness.

Notes: make sure to cool down PFA solution before adding GA.

3.2.2 Harvesting and Tissue Fixation

Day1: Fixing Plant Samples

1. Harvest *Medicago truncatula* nodules at the desired developmental stage.
2. Place plant tissues into the prepared fixative. Ensure the fixative volume is at least **10× the tissue volume** for effective penetration.
3. Apply a vacuum (~500 mm Hg or ~0.065 MPa) to the samples while keeping them on ice. Hold the vacuum for **20 minutes**, release it slowly, and repeat this process twice. This step removes air pockets and allows the fixative to penetrate the tissues fully.
4. Incubate the samples at **4 °C overnight** (at least 12–16 hours) to allow complete fixation.

Notes: The vacuum time and strength might need to be adjusted depending on the samples that you are working with. Please observe the condition of your sample; a fully penetrated sample should sink to the bottom after the vacuum.

3.2.3 Tissue Dehydration

Day 2: Dehydration

1. Remove the fixative from the tissues using a pipette.
2. Pass the tissues through a graded ethanol series at room temperature (RT) to dehydrate them:
    - 10% ethanol: **30 minutes**
    - 30% ethanol: **30 minutes**
    - 50% ethanol: **30 minutes**
    - 70% ethanol: **30 minutes**
    - 90% ethanol: **30 minutes**
    - 100% ethanol: **1 hour × 3**
3. After the final ethanol step, transfer the samples to a fresh 100% ethanol solution and incubate at **4 °C overnight**.

Note: Ensure the paraplast is melted at 58–60 °C overnight in preparation for the infiltration steps.

3.2.4 Paraffin Infiltration

Day 3–6: Infiltration

1. Remove ethanol from the samples and replace it with Histo-Clear in a stepwise manner:
    - **Day 3:**
        - 3:1 ethanol:Histo-Clear, 1 hour
        - 1:1 ethanol:Histo-Clear, 1 hour
        - 1:3 ethanol:Histo-Clear, 1 hour
        - 100% Histo-Clear, 1 hour × 3
    - Immerse tissues in a 1:3 mixture of paraplast:Histo-Clear and incubate **overnight at 60 °C**.
2. Continue replacing the solution with increasing concentrations of paraplast:
    - **Day 4:** 1:2, 1:1, 3:1 paraplast:Histo-Clear (3 hours each), ending with 100% paraplast overnight at 60 °C.

- **Day 5–6:** Replace with fresh 100% paraplast every 3 hours, incubating overnight at 60 °C.

Note: Always ensure sufficient melted paraplast for each infiltration step.

3.2.5 Paraffin Embedding

Day 7: Embedding

1. Pre-warm embedding moulds on a heating plate set to **60 °C**.
2. Stir the tissues gently in melted paraplast to ensure an even coating, then pour the mixture into the mould.
3. Use a needle or forceps to orient the plant tissues for sectioning.
4. Allow the mould to cool on a bench or a cooling plate until the bottom solidifies, then transfer to the ice to speed up solidification.
5. Once fully hardened, remove the paraffin block from the mould and store it at **4 °C** in a sealed bag.

3.3 Paraffin Sectioning and Section Slide Preparation

Day 8: Sectioning

1. **Trim the Paraffin Block:**
   - Using a razor blade, trim the paraffin block to create a trapezoid-shaped cutting surface.
   - Orient the block so that cutting proceeds from the long side of the trapezoid toward the short side. This ensures the ribbon contains adjacent sections of the sample.
2. **Mount the Block on the Microtome:**
   - Secure the paraffin block onto the microtome holder with the longer parallel face positioned at the bottom.
   - Adjust the microtome settings to ensure the block surface is perfectly parallel to the blade.
3. **Cut Thin Sections:**
   - Using a disposable microtome knife, cut sections at a thickness of **8–10 µm**.

- Carefully collect the sections into ribbons. Ensure the shiny (smooth) side of the ribbons faces down.

Note: section thickness might need to be adjusted based on tissue types. For Xenium analysis, the ideal section thickness is between 5 μm to 10 μm.

4. **Collect and Store Ribbons:**
   - Place the paraffin ribbons in a clean paper box to keep them dust-free and organized until mounting.

Day 8: Mounting Sections on Slides

5. **Select Sections for Mounting**
   - Examine the paraffin ribbons under a dissecting microscope to identify **high-quality sections** suitable for mounting.
6. **Stretch Sections on Slides**
   - Apply a **drop of autoclaved and de-gassed MQ water** to the center of a clean glass slide.
   - Transfer the selected sections into the water droplet on the slide. The water will stretch and flatten the sections.
   - Place the slide on a **42 °C hot plate** to assist in further flattening the sections. Typically, **4 sections** can be placed on a single slide.
7. **Dry the Slides**
   - Tilt the slides gently to remove excess water.
   - Allow the tissue sections to dry until they appear opaque, ensuring no water remains on or under the sections.
   - Place the slides in a slide drying rack and incubate for **3 hours at 42 °C** in an oven or on a heating plate.
   - Transfer the slides to a desiccator and leave them to dry overnight at **room temperature** for complete drying.
8. **Storage and Further Use**
   - After overnight drying, proceed with the **deparaffinization and decrosslinking protocol**.

- Alternatively, store the slides with dry tissue sections at **room temperature in a desiccator** for up to **4 weeks**.
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3.3.1 Optional Tissue Block Trimming & Scoring

3.3.2 Paraffin sectioning

Section nodules into thin slices (5, 8, 10 µm) using a microtome.

3.3.3 H&E Staining for Quality Check

3.3.4 Section Placement on Xenium Slides

3.4 Probe Hybridization and Processing Xenium Slides

3.4.1 Deparaffinization & Decrosslinking

3.4.2 Probe Hybridization

3.4.3 Post Hybridization Wash

3.4.4 Ligation

3.4.5 Amplification

3.4.6 Post Amplification Wash

3.4.7 Autofluorescence Quenching

3.4.8 Nuclei Staining

3.5 Post-Xenium Processing

3.5.1 Post-Xenium H&E Staining

3.5.2 Post-Xenium Confocal Imaging

3.5.3 Plant Cell Image Segmentation

3.5.4 Plant Cell Transcript Realignment

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4 Notes

1. Probe Design Considerations:

Gene selection is crucial for successful Xenium profiling. Avoid genes with very short transcripts (<500 bp), low expression, or extensive isoform overlap. Use reference expression datasets to prioritize genes showing tissue- or stage-specific enrichment.

2. Fixative Freshness:

Paraformaldehyde fixative should be prepared fresh each time for optimal crosslinking efficiency. Always cool the PFA solution on ice before adding glutaraldehyde to prevent degradation.

3. Vacuum Infiltration Efficiency:

Apply and release the vacuum gradually to prevent tissue damage. Monitor whether tissues sink—this indicates successful infiltration. Adjust vacuum time based on tissue density or species.

4. Tissue Orientation During Embedding:

Proper orientation is critical for downstream sectioning. When embedding root or nodule samples, place them vertically to maximize the number of longitudinal sections obtained per block.

5. Section Thickness Optimization:

Xenium performs best on 5–10 µm sections. Thicker sections (>10 µm) may compromise probe penetration, while thinner sections may lead to structural collapse or tissue loss during processing.

6. Ribonuclease-Free Handling:

Always use autoclaved or filtered MQ water, RNAse-free tips, and clean slides to reduce contamination. Use gloves and avoid talking directly over open slides during tissue mounting.

7. Section Mounting Tips:

When flattening sections in MQ water, avoid overheating. A 42 °C hotplate helps prevent tissue shrinkage or distortion. Let slides dry fully before processing.

8. Slide Storage Before Hybridization:

Store dried slides in a dust-free, low-humidity environment such as a desiccator. Slides can be stored up to 4 weeks, but longer storage may reduce signal strength.

9. Autofluorescence Quenching:

Plant tissues, especially root nodules, often exhibit high autofluorescence. Always include quenching steps prior to imaging to improve signal-to-noise ratio.

10. Quality Control (QC) with H&E or DAPI:

Before proceeding with expensive hybridization steps, perform H&E or DAPI staining to ensure tissue integrity and RNA preservation. This step also helps in adjusting section thickness.

11. Tissue Compression During Sectioning:

If sections appear wrinkled or compressed, ensure the microtome blade is sharp and the paraffin block is at the correct cutting temperature. Chilling the block slightly before sectioning can help.

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Acknowledgements

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Figure Captions:

Figure 1. Materials for tissue processing and embedding in paraffin blocks.

Figure 2. Positioning of nodule sections on a slide.

Figure 3. H&E section