Xenium In Situ Multimodal Cell Segmentation: Workflow and Data Highlights

Introduction

Cell segmentation is the process of identifying the precise boundary of every cell in an image. The current Xenium In Situ Gene Expression workflow utilizes DAPI staining to perform cell segmentation via nuclear expansion. The Xenium In Situ Gene Expression with Cell Segmentation Staining workflow offers a single solution to more accurately segment cells by utilizing four channels and deep learning algorithms trained on Xenium data.

This Technical Note summarizes the Xenium In Situ Gene Expression with Cell Segmentation Staining workflow and multimodal cell segmentation algorithm, including several data highlights. Best practices and mitigation strategies are also provided for common issues.

Cell segmentation is also a mandatory component of the Xenium In Situ Gene and Protein Expression assay, which is compatible with human FFPE samples. Though the data shown in this Technical Note is specific to the Gene Expression-only assay, the background and conclusions apply to both assays.

Overview

The Xenium In Situ Gene Expression with Cell Segmentation workflow utilizes a multimodal approach to segment cells. Staining is performed with a single cocktail of Xenium Multi-Tissue Stain Mix (PN-2000991). The mix provides four different labeling methods that serve as inputs for automated cell segmentation: antibodies labeling membranes, antibodies labeling cell interior, a universal interior label against Ribosomal RNA, as well as the nuclear label DAPI (Figure 1). Together, this Xenium Multi-Tissue Stain Mix provides cell boundary-based segmentation for cells with good membrane labeling, and interior stain-based labeling or nuclear expansion to define boundaries when membrane labeling is less informative.

The cell segmentation staining workflow is fully integrated into both the Xenium In Situ Gene Expression and Gene and Protein Expression assays (Figure 2). There is an extra day of in-lab work, including an overnight incubation. Samples stained for cell segmentation are run on the Xenium

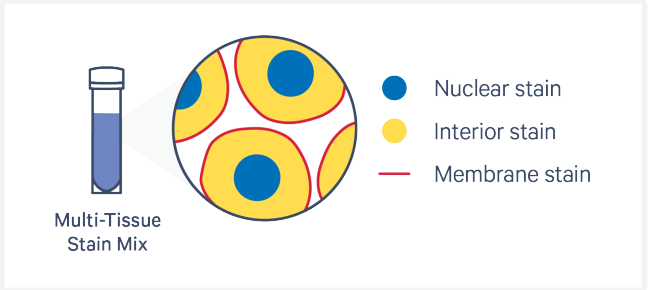


Figure 1. The Xenium In Situ Gene Expression with Cell Segmentation Staining assay uses a Multi-Tissue Stain Mix for broad coverage of tissues and cell types.

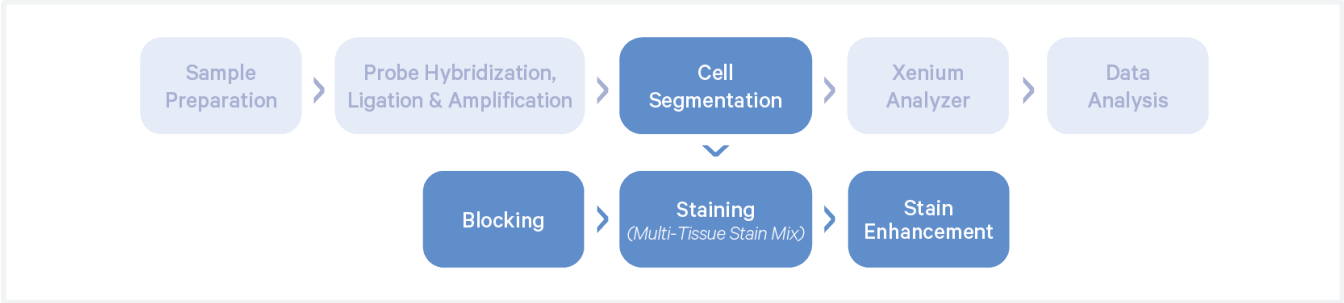


Figure 2. A representation of the Xenium Cell Segmentation Staining workflow within the broader Xenium workflow.

Analyzer instrument, where the data is simultaneously collected and processed by the Xenium Onboard Analysis pipeline. See below for a summary of the workflow, timing, and algorithms.

The assay is compatible with both FFPE and fresh frozen tissues and is validated for human and mouse tissue types. The workflow is executed using the provided Xenium Cell Segmentation Staining Reagents Kit (2 rxns, PN-1000661). The Xenium Cell Segmentation Detection Reagents Kit (PN-1000639) is used for running the samples on the Xenium Analyzer instrument. See the Xenium In Situ Gene Expression with Cell Segmentation Staining User Guide (CG000749) and Xenium Analyzer User Guide (CG000584), respectively, for more information about reagent kits and additional consumables.

Cell Segmentation Staining

The Xenium Multi-Tissue Stain Mix consists of stains for broad-coverage cell segmentation in four channels. These stains target a variety of cell types and tissues. DAPI stain is used for nuclear staining and nucleus segmentation. Antibodies for ATP1A1, E-Cadherin, and CD45 are used for segmenting cell membrane boundaries. They produce clear boundaries of epithelial and immune cells in a majority of tissue types. 18S Ribosomal RNA labels the cytoplasm and is considered a pan-cell type marker. alphaSMA/Vimentin antibodies are included in the cocktail for interior protein staining. They are cytoskeletal proteins that work on a variety of different cell types within the extracellular matrix, mainly targeting fibroblasts and smooth muscle cells. Vimentin can be expressed in the cytoplasm and cell membrane.

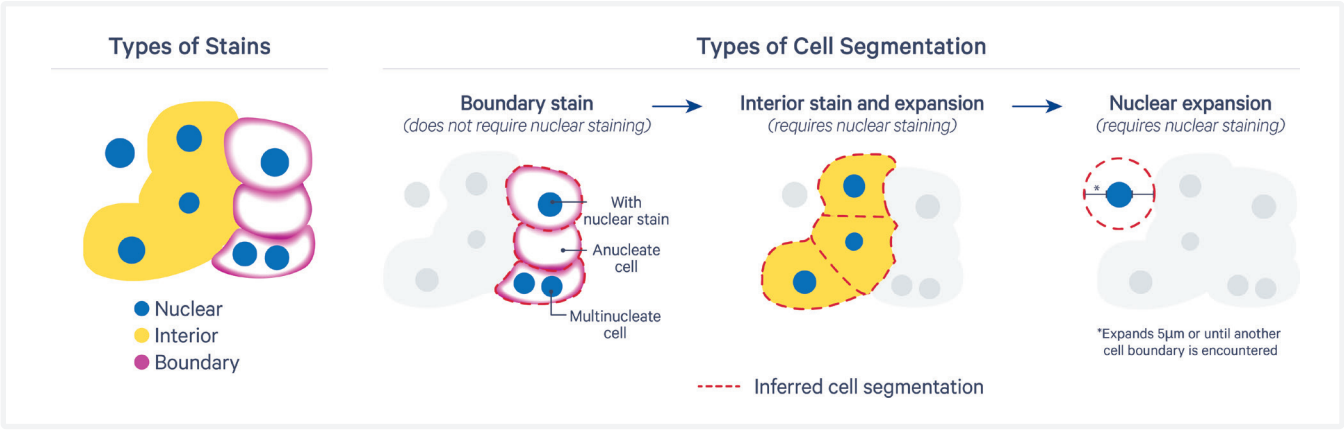


Figure 3. A representation of the Xenium multimodal cell segmentation algorithm.

Algorithm & Software

All references to the Xenium In Situ software suite in this Technical Note refer to v2.0 and later. The Xenium In Situ software suite has been updated to v2.0 for Xenium Onboard Analysis, Xenium Explorer, and Xenium Ranger. This version enables multimodal cell segmentation and downstream analysis. In addition, the default isotropic nuclear expansion distance is 5 µm in Xenium Onboard Analysis for both cell segmentation algorithms (nuclear expansion-only and multimodal cell segmentation). Internal studies show the 5 µm expansion distance achieves more accurate transcript assignment compared to the previous default of 15 µm.

The multimodal cell segmentation algorithm uses custom deep learning models trained on Xenium data. The new algorithm has three methods to segment cells. The final segmentation result for each cell is prioritized in this order: by boundary stain, by expansion from nucleus to interior RNA stain edge, or by isotropic nuclear expansion of 5 µm (Figure 3).

The multimodal cell segmentation algorithm can detect cells with two or more nuclei in one cell using the boundary stain method. While the majority of cells contain one nucleus, some cells possess two or more nuclei. Examples of multinucleate cells with normal biological function include osteoclasts, hepatocytes, and skeletal muscles. Cell types associated with cancers or other pathologies may also contain multiple nuclei per cell. Additionally, the boundary method can segment cells with boundary stain but no nuclei.

The Xenium Onboard Analysis algorithm gathers intracellular information from the 18S marker. AlphaSMA/Vimentin has a broad staining pattern (can include membrane and intracellular regions) that overlaps with 18S. Given the overlap and to simplify model training, the multimodal cell segmentation algorithm does not currently use the interior protein stain. However, the interior protein stains are useful for qualitatively assessing that segmentation results corroborate with the morphology of the cell types in the tissue sample.

Xenium Onboard Analysis v2.0 includes changes to output files to accommodate the addition of multi-tissue stain image processing and multimodal cell segmentation. The analysis summary includes "Nuclear transcripts per 100 µm2" in Key Metrics and the Decoding tab to enable comparing results regardless of cell segmentation method. The Cell Segmentation tab includes the proportion of cells segmented by either boundary stain, interior RNA, or nuclear expansion. If fewer than 50% of cells are segmented by boundary and interior RNA methods, it is a sign that staining was suboptimal or the sample is potentially unsuitable for the multi-tissue stain. A suboptimal stain may indicate a workflow or instrument error, or poor sample quality. Data can be rerun with the multimodal cell segmentation algorithm in Xenium Ranger, which supports removing suboptimal stains and adjusting the default nuclear expansion distance.

Xenium Explorer enables visualization of multi-tissue stain and post-Xenium images with cells colored by segmentation method (Figure 4). Since each cell is labeled by its segmentation method, the software can help with quality checking segmentation results. Note that Xenium Explorer displays segmentation polygon boundaries, which are approximations for visualization performance. The true nucleus and cell segmentation mask results are available in the cells.zarr.zip output file.

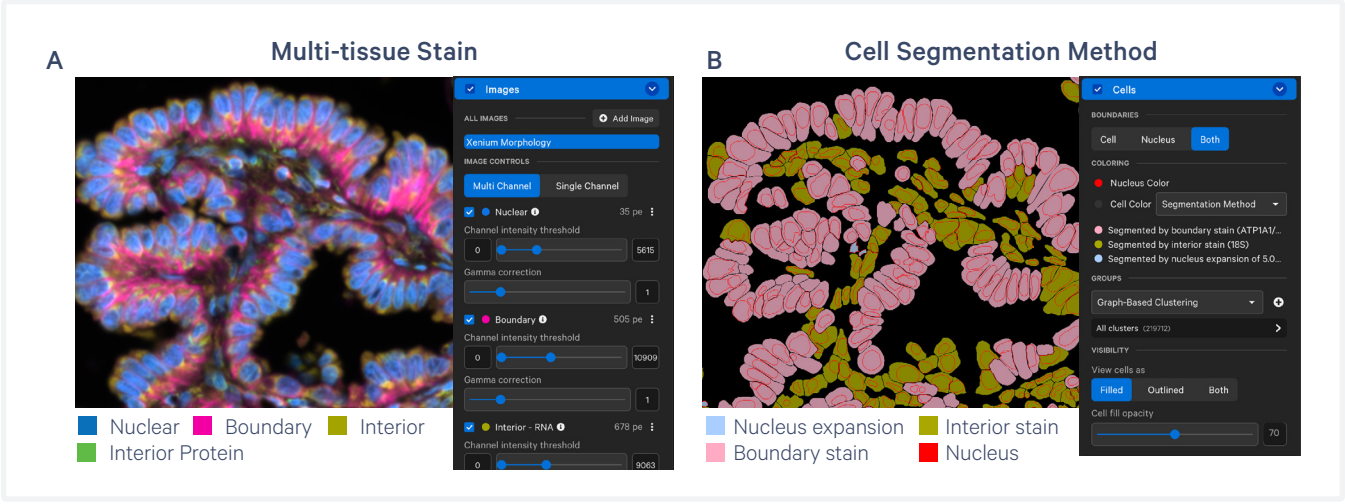


Figure 4. Visualization of **(A)** multi-tissue stain and **(B)** cells by segmentation method (nuclei outlines: red, boundary stain method: pink, interior RNA method: yellow, nuclear expansion method: blue) for a region of human lung tissue in Xenium Explorer v2.0.

Results - Data Highlights

Multi-tissue stain and multimodal cell segmentation solution perform in a range of tissues

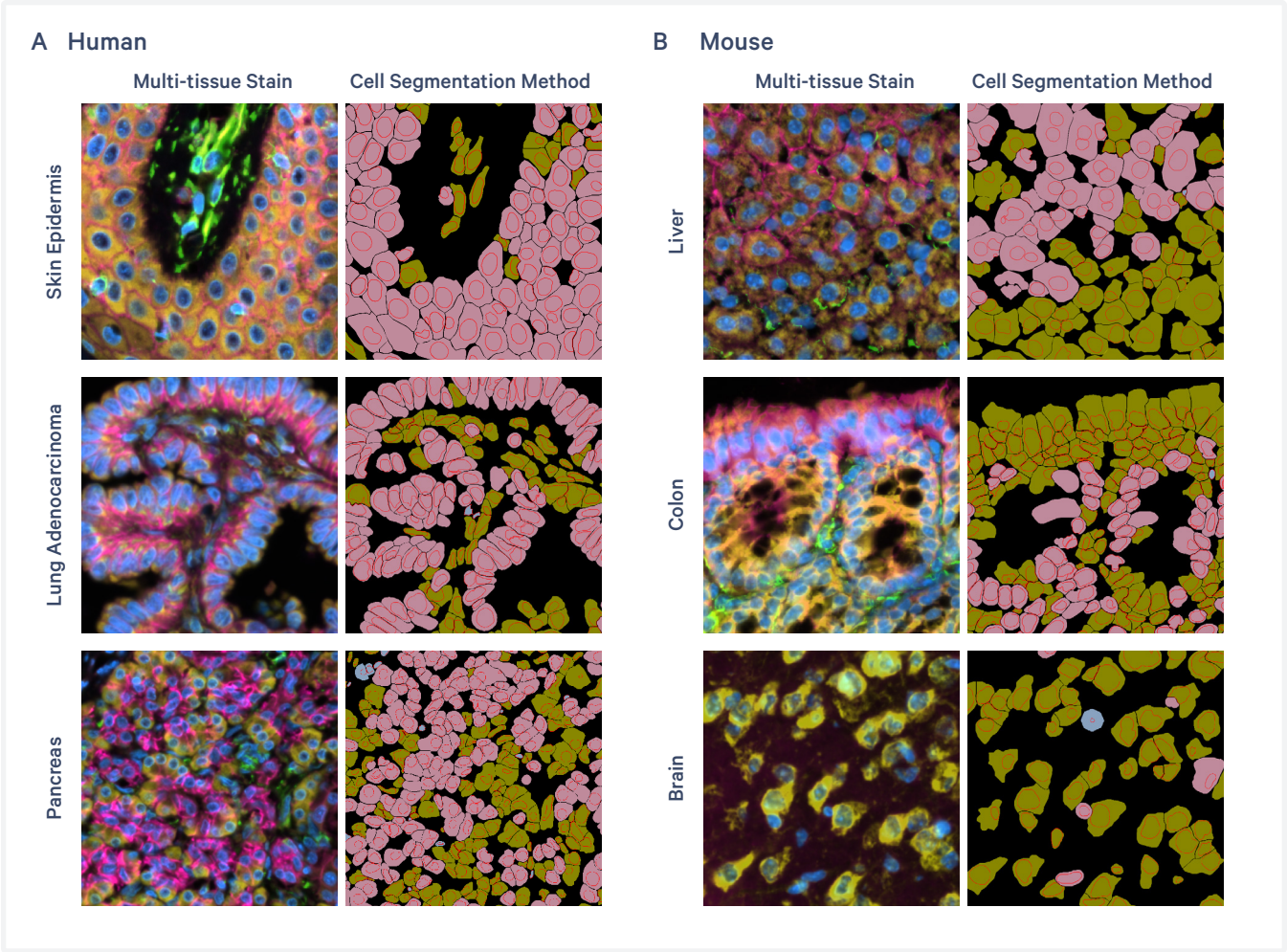


Figure 5. Examples of **(A)** human FFPE and **(B)** mouse fresh frozen multi-tissue stain images and multimodal cell segmentation results for a variety of tissues and cell types. The segmentation results are visualized with Xenium Explorer v2.0. Stain and segmentation colors are the same as in Figure 4.

Assay sensitivity is comparable with or without cell segmentation staining workflow

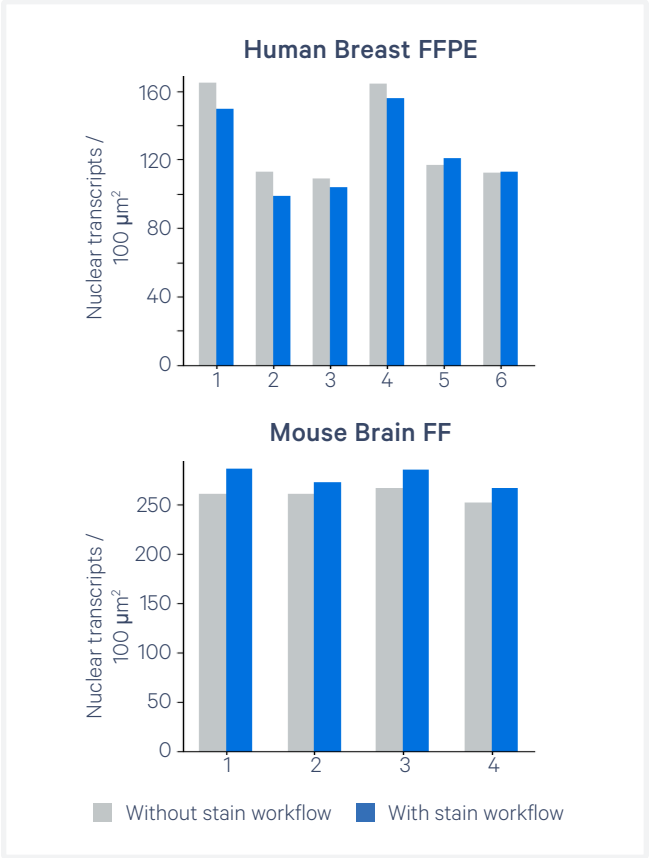


Figure 6. There is little to no change in assay sensitivity between the existing workflow (CG000582) and the extended workflow with added cell segmentation staining steps (CG000749). Xenium Onboard Analysis v2.0 was used for both analyses.

To compare transcript assignment results from these assays, sensitivity was measured by the number of nuclear transcripts per 100 µm². Several replicates for FFPE human breast and fresh frozen mouse brain samples are shown with tissue-specific pre-designed gene panels to demonstrate the small differences in this metric between workflows.

This metric is not influenced by differences in cell size resulting from either the nuclear expansion or multimodal cell segmentation algorithms. It is provided in Xenium Onboard Analysis v2.0 analysis and metrics summary outputs.

Transcript assignment with multimodal cell segmentation

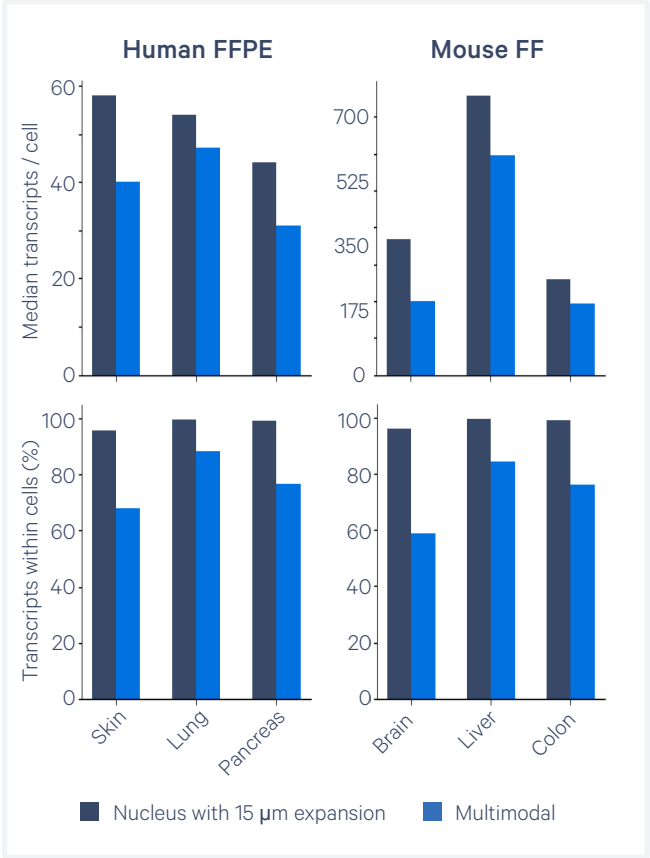


Figure 7. Comparison of transcript assignment metrics for several human and mouse tissue types using the cell segmentation staining workflow. The data were analyzed with either isotropic nuclear expansion (15 µm) or multimodal cell segmentation algorithms.

The percent of transcripts within cells and median transcripts per cell decrease when the data is analyzed with multimodal cell segmentation. This trend is mainly driven by the overestimated cell size in the 15 µm nuclear expansion method. In the multimodal cell segmentation approach, there is now more intercellular space, which better agrees with tissue morphology and biology.

More accurate cell morphology with multimodal cell segmentation

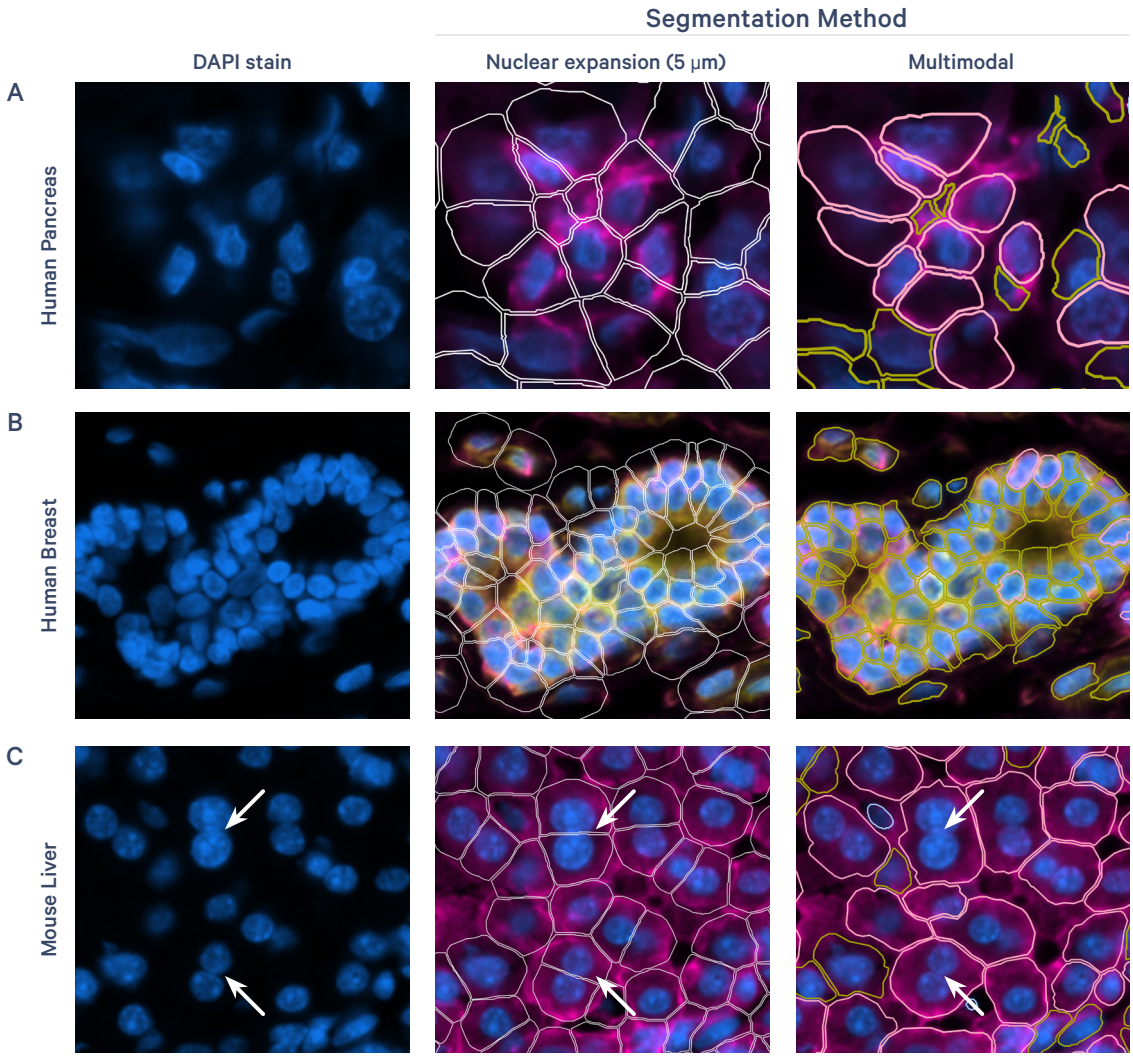


Figure 8. Visual comparison of cell boundaries generated by multimodal cell segmentation methods (yellow, pink, blue polygons) vs. 5 µm isotropic nuclear expansion (white polygons). The cells segmented by the boundary or interior stain methods more accurately match different cell morphologies, while the isotropic nuclear expansion method alone produces rounder boundaries. Examples demonstrate: A) cells from human pancreas with improved cell boundaries between adjacent cells, B) a variety of cell types in healthy human breast tissue with improved cell boundaries at the cell periphery, and C) dense and multinucleate cells in mouse liver (indicated by white arrows) that are identified by the boundary stain method. Binucleate cells are expected in dense tissues such as liver.

The nuclear expansion-only analysis uses DAPI for cell segmentation, while the multimodal cell segmentation method uses DAPI, boundary, and interior stains. The 5 µm nuclear expansion cell boundaries (white) are overlaid on the multi-tissue stain images for comparison with the multimodal results, however these boundaries were generated with DAPI information only.

Improved cell type assignment with multimodal cell segmentation

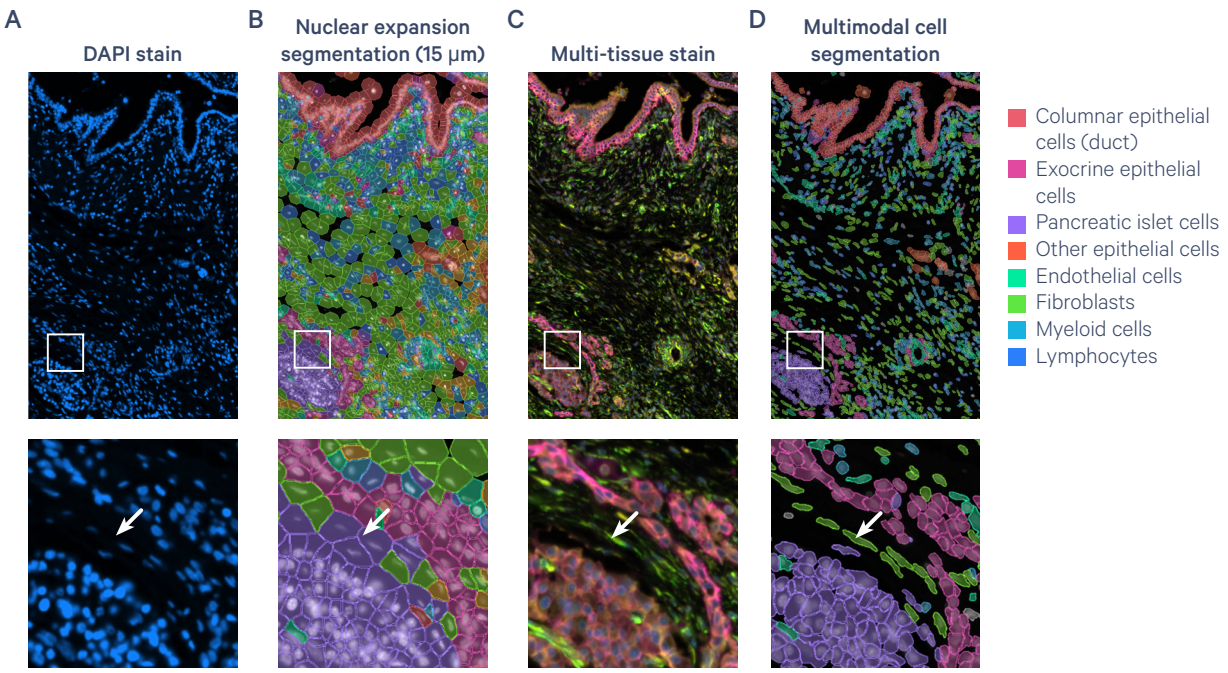


Figure 9. More accurate cell typing of fibroblast niche cells with multimodal cell segmentation. A) The DAPI nuclear stain image zoomed into a region of human pancreas. B) Cell segmentation with 15 µm isotropic nuclear expansion results and cells colored by major cell types determined from annotating unsupervised clusters (legend to the right of the images). C) Multi-tissue stain image showing multiple common pancreatic cell types (per-channel DAPI, boundary, interior RNA, and interior protein colors are the same as in Figure 4A). The alphaSMA/Vimentin stain (green; white arrow) shows the elongated shape of the fibroblast cells. D) Same cell type analysis as B), but with multimodal cell segmentation results, which have a 5 µm expansion distance if the nuclear expansion method is used. Some fibroblasts are incorrectly classified as pancreatic islet cells (purple; white arrow) in the 15 µm analysis (B). This is due to misassigning highly expressed transcripts from neighboring islet cells to the fibroblast cells of interest. Panel D shows the improved cell segmentation results where the fibroblast-like elongated morphology is now visible (light green; white arrow).

Additional Considerations

Many factors may influence the quality of cell segmentation data, including:

- **Tissue quality**: poor tissue quality and/or preparation
- **Tissue type:** some tissues, including dense tissue types, may be prone to more background or may be more difficult to segment. See 10x support site for Multimodal Cell Segmentation Validated Tissue List.
- **Cell type:** elongated cells, such as muscle cells, and adipocytes are challenging to segment. See 10x support site for Multimodal Cell Segmentation Validated Tissue List.
- **Tissue fixation:** overfixation may lead to weaker staining
- **Sectioning mistakes:** variation in tissue thickness within a section may lead to uneven staining
- **Storage conditions:** improper storage of tissue samples may lead to weaker stains or artifacts
- **Workflow errors:** if the Xenium Multi-Tissue Stain Mix is not centrifuged, antibody aggregates may bind to the tissue and lead to stain artifacts
- **Suboptimal staining:** multinucleate and anucleate cells will not be detected in the absence of good boundary stain

To avoid issues with cell segmentation, the following **best practices** are recommended:

- Pilot study of challenging tissue types is recommended
- Process samples according to best practices. In particular, follow the recommended fixation times for FFPE tissue preparation.
- Practice sectioning before using experimental samples
- Store tissue samples properly. Consult Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578) or Xenium In Situ - Fresh Frozen Tissue Preparation Handbook (CG000579) for additional information about tissue storage
- Follow the protocol carefully for recommended tips & best practices
- Keep the lab area clean and free of lint and dust when possible

Importantly, the Xenium In Situ Gene Expression with Cell Segmentation Staining Workflow is compatible with all post-Xenium applications, including H&E, IF, and Visium CytAssist Spatial Gene Expression. Because cell segmentation antibodies in the Xenium Multi-Tissue Stain Mix are raised in rabbit, do not use anti-rabbit secondary antibody for post-Xenium applications as it will result in cross-detection. For all post-Xenium applications after cell segmentation staining, use primary antibodies that are not raised in rabbits. If using a primary antibody raised in rabbit, ensure that it is directly conjugated to a detection tag (such as a fluorescent tag or biotin), thereby negating the use of a secondary anti-rabbit antibody for detection. See the Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression Technical Note (CG000709) for additional guidance.

Conclusions

This Technical Note demonstrates that cell segmentation staining assay sensitivity is comparable to data generated without the stain mix and that while transcript assignment metrics are reduced, it is largely the result of more biologically accurate cell segmentation results. In conclusion, Xenium multimodal cell segmentation offers comprehensive cell segmentation staining in four channels. Advantages of Xenium's multimodal cell segmentation workflow include:

- Fully integrated with Xenium In Situ workflow
- Transcript and stain images are collected in a single instrument run, minimizing mismatches due to tissue warping
- Compatible with FFPE and fresh frozen tissue, validated in both human and mouse
- The segmentation algorithm is designed and optimized for Xenium data
- Bases cell segmentation on biological features for more accurate transcript assignment
- Enables detection of some multinucleate and anucleate cells

References

1. Xenium In Situ Gene Expression with Cell Segmentation Staining User Guide (CG000749)
2. Xenium Analyzer User Guide (CG000584)
3. Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578)
4. Xenium In Situ - Fresh Frozen Tissue Preparation Handbook (CG000579)
5. Xenium In Situ Gene Expression User Guide (CG000582)
6. Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression Technical Note (CG000709)

Datasets

www.10xgenomics.com/datasets

Document Revision Summary

Document Number	CG000750
Title	Xenium In Situ Multimodal Cell Segmentation: Workflow and Data Highlights Technical Note
Revision	Rev B
Revision Date	August 2025
Description of Changes	Added information on the Xenium Gene and Protein Expression workflow on page 1. Updated references for Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578) throughout. Updated references for Xenium In Situ - Fresh Frozen Tissue Preparation Handbook (CG000579) throughout. Updated for general minor consistency of language, format, and terms throughout.

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