Shedding New Light On **MICROSCOPY**

APPLICATION NOTE

ECLIPSE Ti2 Microscope
AX/AX R with NSPARC Confocal-based Super Resolution Microscope

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NSPARC cyclic imaging for super-resolution spatial multi-omics

Imaging technologies have transformed spatial biology, particularly spatial transcriptomics. Visualizing the transcriptional states of cells in situ while retaining spatial context is a powerful way to understand disease and uncover new biology. Combining high-resolution imaging with direct access to raw image data further improves data quality and allows the information contained within samples to be used to the fullest.
Here, we used Nikon's NSPARC super-resolution confocal imaging technology and an advanced multiplexed workflow spanning ultraviolet (UV) to near-infrared (NIR) wavelengths to identify single RNA transcripts in tissue samples. In this study we used multiplex *in situ* hybridization to analyze 16 astrocyte-specific genes in fresh-frozen mouse brain tissue, although the workflow is compatible with a broad range of fluorescence staining approaches.
Using ROI-based targeted imaging, integration with multimodal spatial omics and analysis while retaining access to raw image data, we visualized transcripts in three dimensions at subcellular resolution. We also demonstrated accurate assignment of transcripts to astrocytes, despite their complex cellular morphology.
Overall, this system provides a 3D super-resolution spatial multi-omics platform covering the UV-to-NIR range. By making full use of the microscope's imaging capabilities, it enables spatial biology analyses with high precision and high information content.

Keywords: NSPARC; super-resolution microscopy; UV–NIR; cyclic imaging; multi-omics; 3D imaging.

Image-based spatial transcriptomics analysis

The microscope described here supports a broad spectral range from UV to NIR point-scanning confocal microscopy and NSPARC super-resolution confocal imaging in a single multimodal system. This configuration enables a wide variety of fluorescence-based assays to be carried out flexibly within one workflow.

Imaging began with a whole-mouse-brain overview acquired using a low-magnification objective (Plan Apo λD 10X). This overview served as the reference image for selecting regions of interest (ROIs) for detailed analysis (Figure 1a). The selected ROIs were then imaged with a higher magnification objective (Plan Apo λD 20X), a resonant scanner and the NSPARC SPAD array detector. This setup enables acquisition over large areas while maintaining the sensitivity required to detect fluorescent spots from individual RNA molecules.

For RNA analysis, we applied a cyclic imaging workflow in which staining, imaging and de-staining were repeated. Four analysis cycles were performed. In each cycle, nuclei were imaged in the UV channel and RNA transcripts were detected using four fluorescence channels. By using the system's broad spectral coverage from UV to NIR, we achieved high multiplexing performance together with strong spectral separation, enabling efficient multi-gene analysis.

Figure 1 shows the post-acquisition analysis workflow. The acquired tile images were stitched (Figure 1b), followed by image registration between cycles (Figure 1c), cell segmentation and spot detection (Figures 1d,e). This produced a cell-by-gene matrix assigning detected transcripts to individual cells, which was visualised alongside the DAPI image (Figure 1f). The resulting data can then be used for downstream bioinformatics analysis.

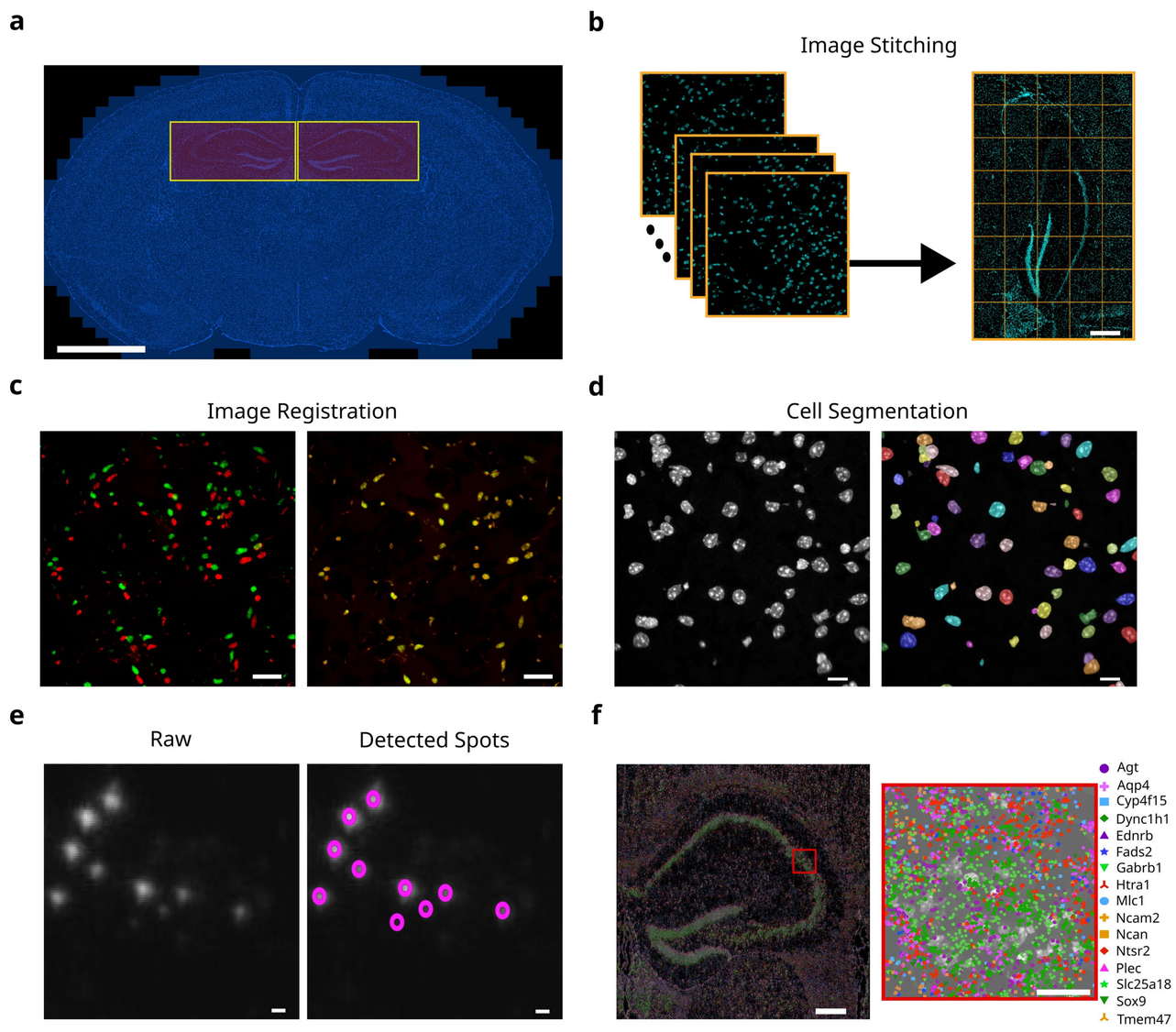

Figure 1: Image processing of NSPARC spatial transcriptomics data.
(a) Whole-sample overview and selected ROI acquired by epifluorescence imaging using a 10X objective (PLAN APO λD). Scale bar: 2 mm. (b) Tile images stitched to generate the dataset for subsequent processing. Scale bar: 500 µm. (c) Registration of stitched images from different imaging rounds to correct inter-round offsets. Scale bar: 50 µm. (d) Nuclear segmentation using Cellpose. Scale bar: 50 µm. (e) Spot detection in a representative region using a deep learning-based method. Scale bar: 1 µm. (f) Decoded transcripts mapped to the selected ROI. Scale bars: left, 500 µm; right, 100 µm.

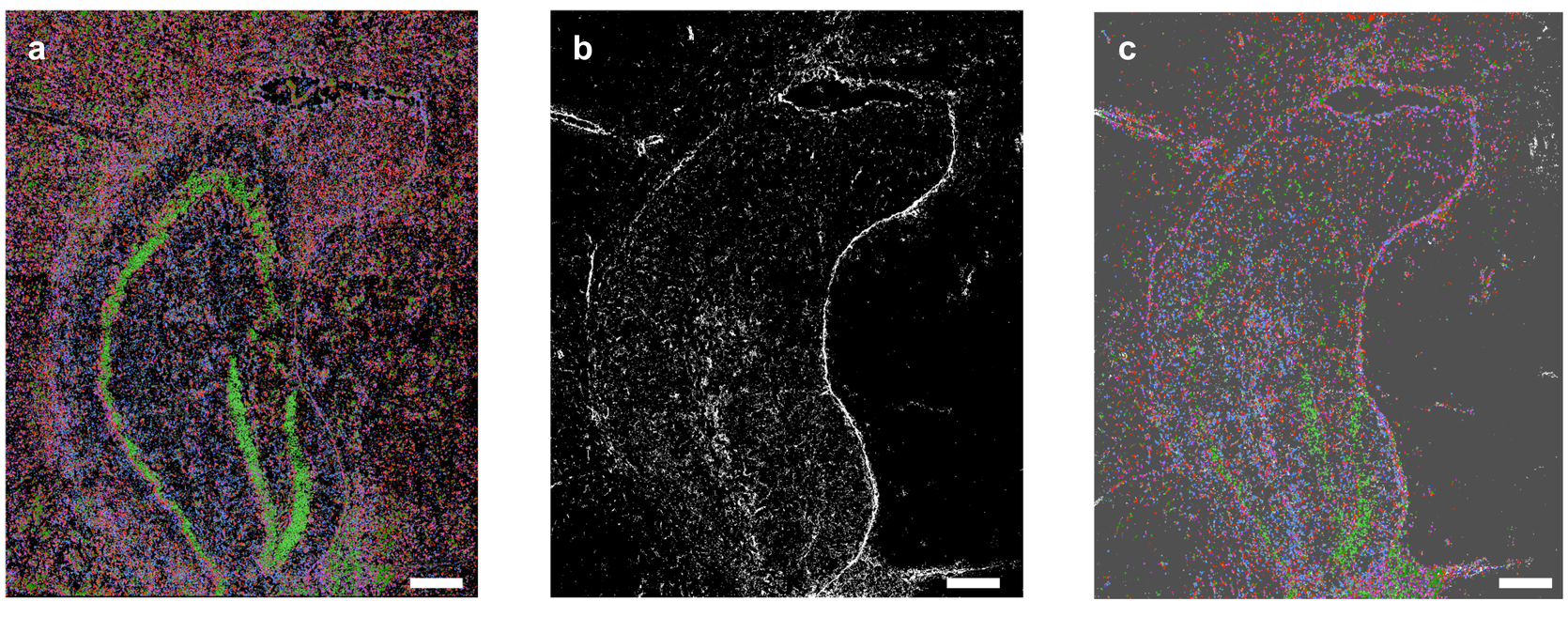

Figure 2: Multi-omics combining immunofluorescence (IF) and spatial transcriptomics.
(a) Decoded transcripts overlaid on the DAPI image. Scale bar: 500 µm. (b) Immunofluorescence-derived mask showing GFAP-positive cells. Scale bar: 500 µm. (c) Subset of decoded transcripts associated with the IF mask, demonstrating integration of protein information and transcript data within the same tissue section. Scale bar: 500 µm.

Multimodal spatial omics

Image-based detection of RNA transcripts while preserving cellular spatial context is a powerful approach for analyzing gene expression within tissues. However, assigning transcripts to individual cells depends heavily on downstream bioinformatics and is vulnerable to artefacts arising from cell segmentation.
To identify astrocytes accurately and achieve more reliable cell segmentation, this experiment incorporated standard immunofluorescence (IF) staining before RNA analysis. Samples were stained with a primary antibody against GFAP, an astrocyte marker, a fluorescently labelled secondary antibody and DAPI. The samples were then de-stained, hybridized with gene-specific probes for the target RNAs and subjected to cyclic imaging through repeated rounds of staining, imaging and de-staining. Four cycles were performed, analyzing four genes per cycle and 16 genes in total.
Integrating the GFAP and RNA signals showed that, as intended by the astrocyte-specific gene panel, the majority of detected RNA molecules were localized within astrocytes (Figure 2). This demonstrates that combining immunofluorescence information with spatial transcriptomics data enables cell-type-specific gene expression to be captured with high accuracy.

Taking advantage of the system's flexibility, we also observed individual cells in detail using a high-magnification oil-immersion objective. Using the IF-derived cell mask enabled transcripts to be assigned to each cell with greater confidence (Figure 3a). In practice, transcripts may belong to the same cell even when they are located away from the nucleus (Figure 3a, arrows), while transcripts close to a nucleus may originate from a different cell (Figure 3a, arrowheads). Accurate attribution therefore requires segmentation that reflects the complex morphology characteristic of astrocytes.
Overall, these results show that multi-omics analysis incorporating immunofluorescence information enables accurate transcript assignment even in cells with complex morphology, improving the reliability of spatial transcriptomics analysis.

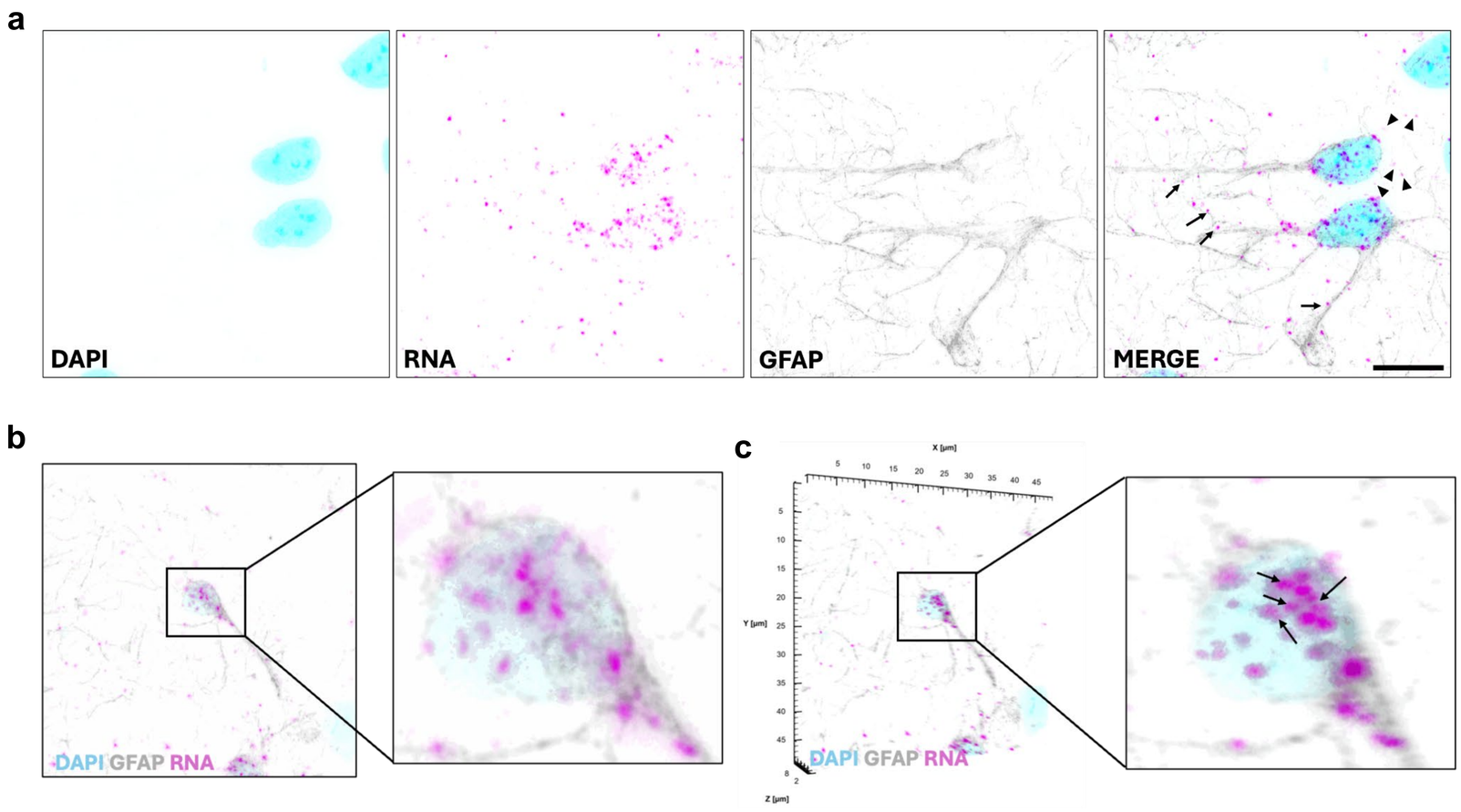

Figure 3: Multimodal and 3D spatial transcriptomics with NSPARC.
(a) Astrocyte image. Cyan: nuclei (DAPI); magenta: RNA transcripts (Alexa Fluor 647); grey: GFAP (Alexa Fluor 568). Arrows indicate transcripts located within GFAP-positive astrocyte processes away from the nucleus, while arrowheads indicate transcripts near a nucleus but not associated with that cell. Scale bar: 10 µm. (b) Maximum intensity projection of an astrocyte; (c) 3D image. 3D rendering resolves individual transcripts (arrows) that appear to overlap in projection images because they are separated along the z-axis. Images were acquired with the AX NSPARC detector and a PLAN APO λD 60X Oil objective (resonant scanner, 2048 × 2048 pixels, 6× zoom).

Super-resolution subcellular 3D transcriptomics with NSPARC

To evaluate NSPARC 3D super-resolution imaging performance, we carried out three-dimensional transcript analysis in selected ROIs. Tissue sections in which astrocytes were labelled with an anti-GFAP antibody, and in which all target RNA molecules were visualized, were used as samples. By acquiring 3D image stacks containing DAPI, GFAP and RNA signals, we visualized the subcellular localization of RNA molecules within individual cells (Figure 3b).

The enlarged images reveal the three-dimensional distribution of transcripts within the region of interest in detail. NSPARC super-resolution imaging improved the separation of nearby RNA spots not only within single XY planes, but also along the Z axis (Figure 3c, arrows). This indicates that the spatial arrangement of transcripts within individual cells can be analyzed more accurately. Together, these results demonstrate the feasibility of 3D subcellular transcriptomics analysis on a commercial microscope platform.

Conclusion

In conclusion, this platform is a multimodal imaging system that integrates widefield (WF), confocal and NSPARC super-resolution imaging into a single workflow. This integration allows users to choose the most appropriate imaging modality seamlessly, according to the sample and research question, from rapid large-area screening to high-contrast optical sectioning and visualization of nanoscale structures.
Broadband spectral imaging from UV to NIR also greatly expands the range of usable fluorescent probes and detectable signals, supporting complex multiplexed analyses and custom experimental designs. In combination with cyclic imaging, it delivers high-content, high-throughput imaging while maintaining high spatial resolution and strong spectral separation.

In addition, the acquired raw image data can be stored and exported to major community-standard formats, enabling smooth integration with open platforms for analysis and visualization, such as MC Micro, Spatialdata, Vitessce and TissUUmaps.
Together, these capabilities make the platform a 3D super-resolution spatial multi-omics foundation spanning the UV-to-NIR range, enabling biological complexity to be analyzed with greater detail and breadth than ever before.

Imaging conditions

Epifluorescence images were acquired using a 10X objective (PLAN APO λD) an optical configuration (OC) with illumination source set to 400nm wavelength, and an exposure time of 5ms. The scan area per tile was 2048 x 2048 pixels. Multiple tiles were imaged to cover the whole tissue area.

NSPARC images were acquired using a 20X (or 60X) objective (Plan Apo λD dry or oil respectively), a resonant scanner and the NSPARC SPAD array detector. Zoom was set to 3 (or 6 for 60X). The scan area per tile was 2048 x 2048 pixels. Illumination sources were 405 nm, 488 nm, 561 nm, 640 nm and 730 nm. The NSPARC collections lenses were auto-aligned for all wavelengths before acquisition. All images were acquired using a Z-stack of 7um, with a 1um step. Illumination intensities were set based on experimental conditions.

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Product information

ECLIPSE Ti2 Microscope, AX/AX R with NSPARC Confocal-based Super Resolution Microscope

Combining the ECLIPSE Ti2 inverted microscope with the AX/AX R confocal system and NSPARC super-resolution detector creates a powerful platform for spatial multi-omics.

Its broad field of view enables efficient ROI selection, while the 25-element NSPARC detector delivers the resolution and sensitivity needed to detect individual RNA transcripts across a broad UV-to-NIR spectral range and visualize them in 3D.

Product information: