Highly Multiplexed Immunofluorescence of the Human Kidney using Co-Detection by Indexing (CODEX)

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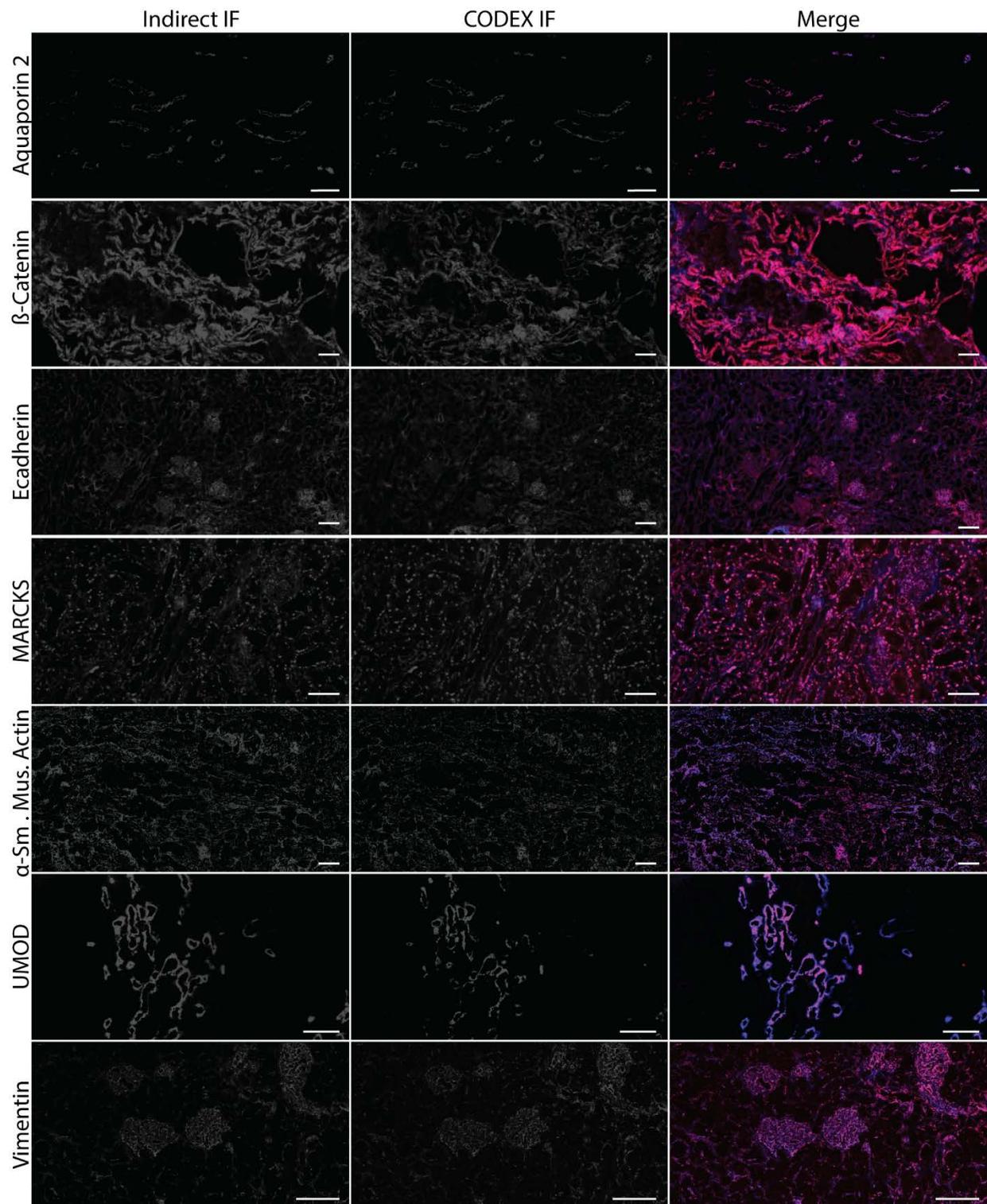
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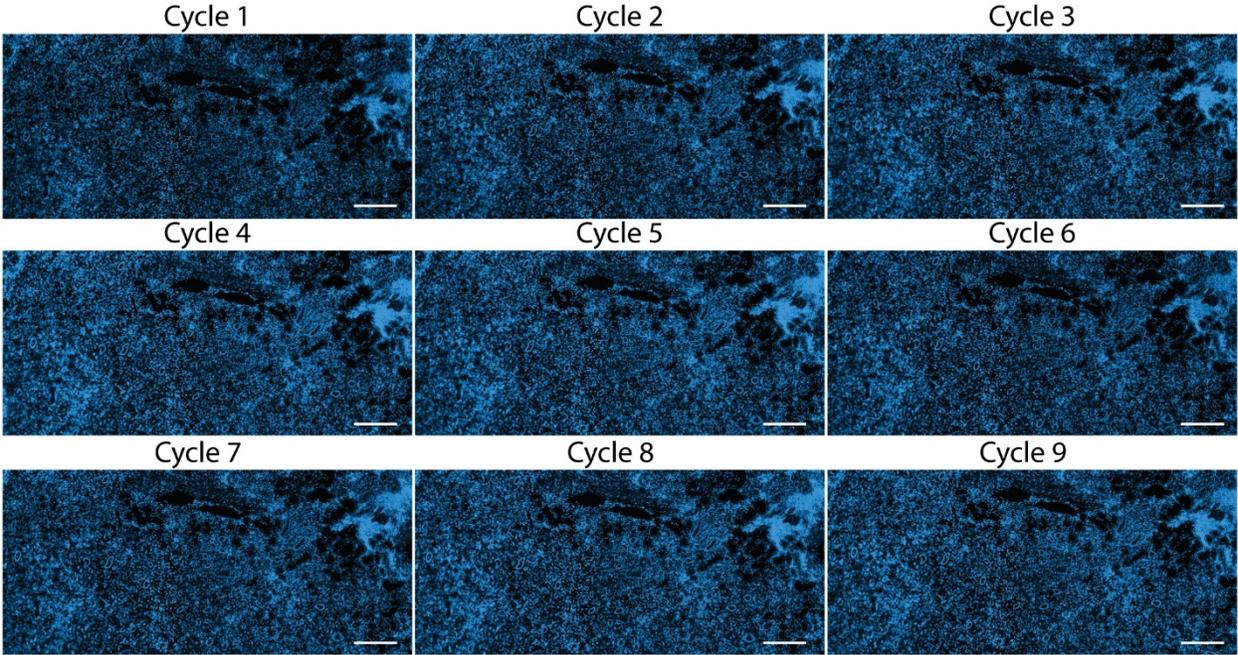
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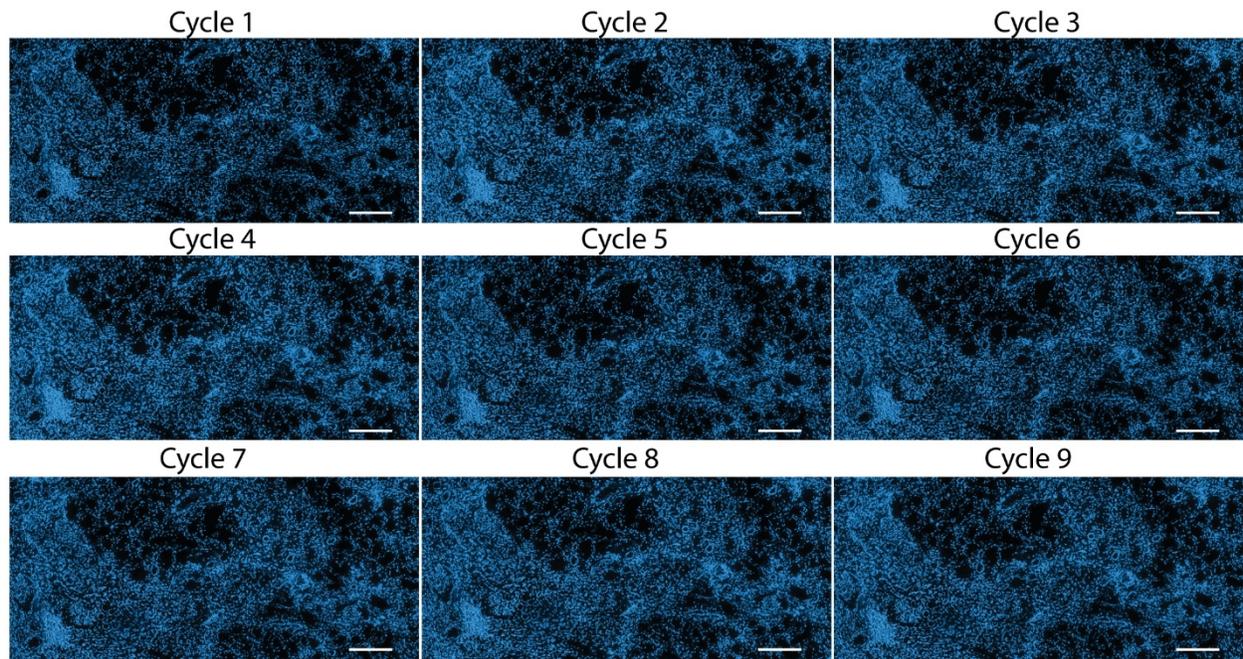
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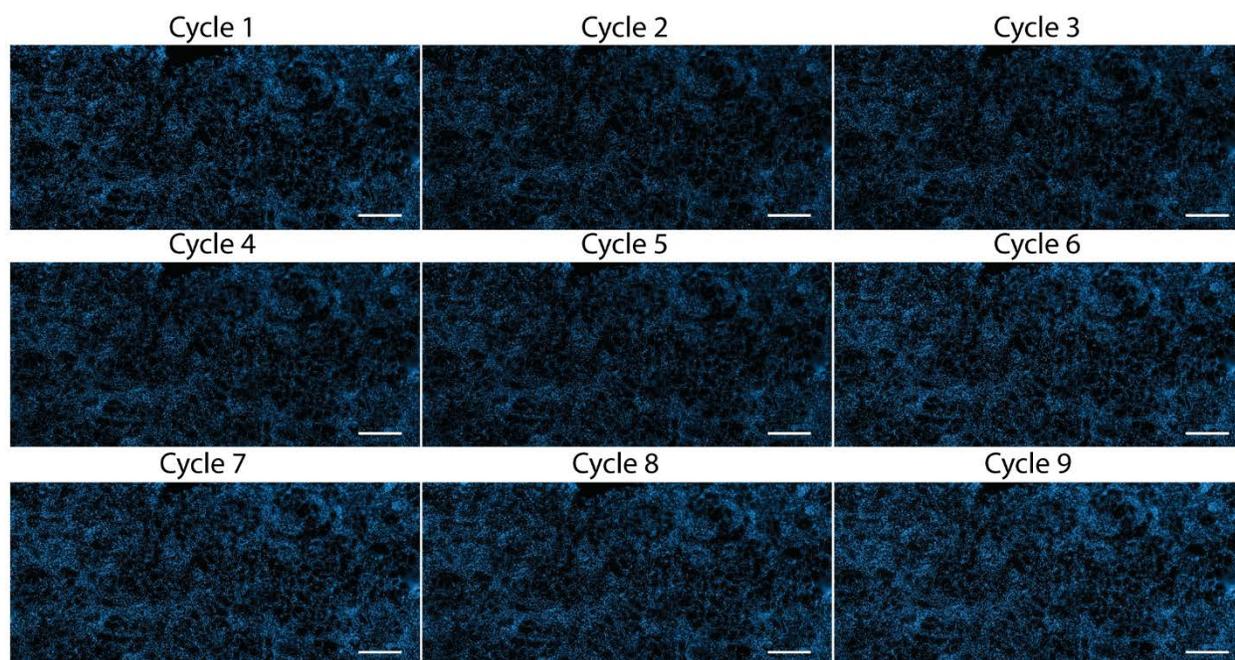
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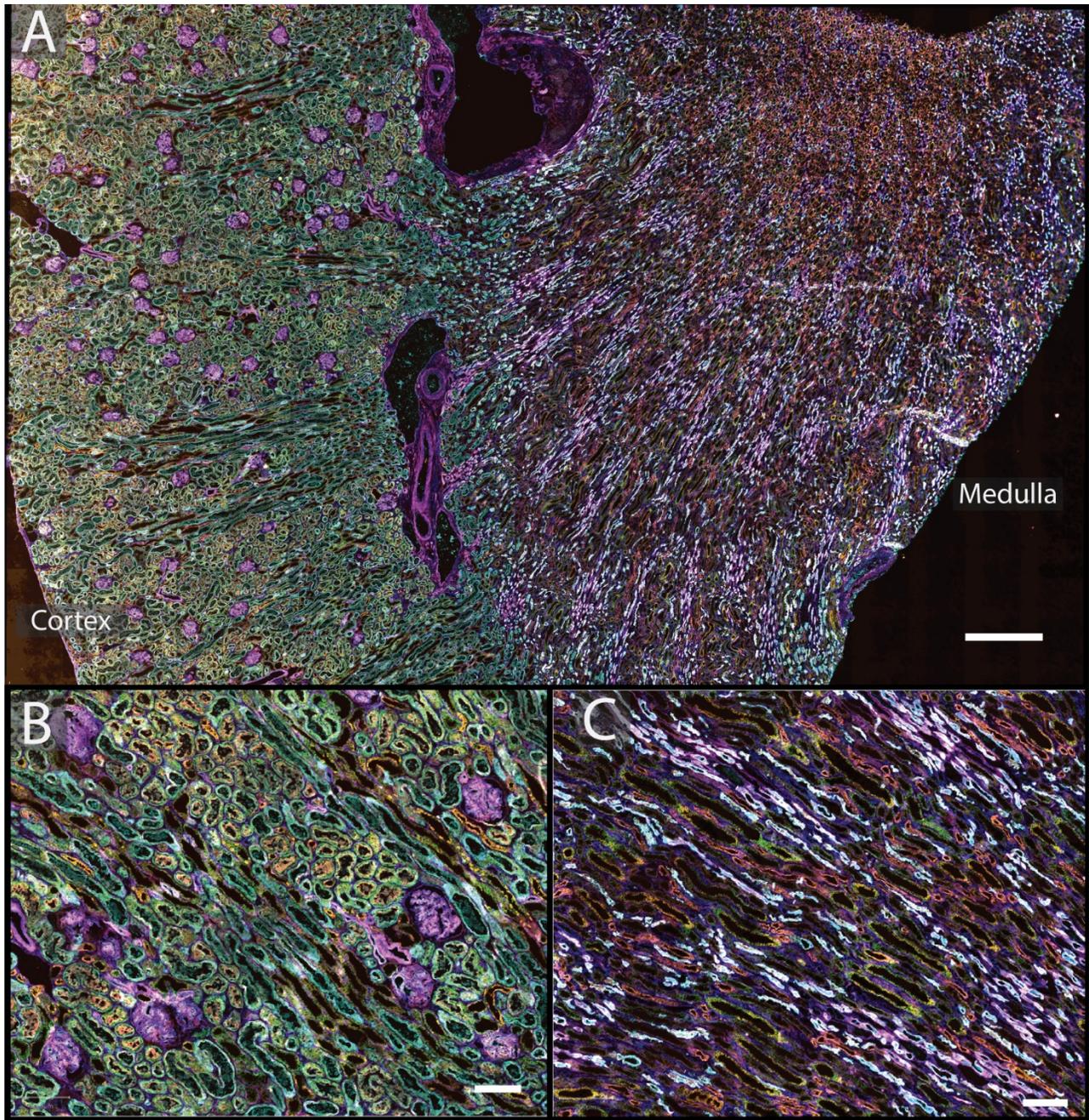
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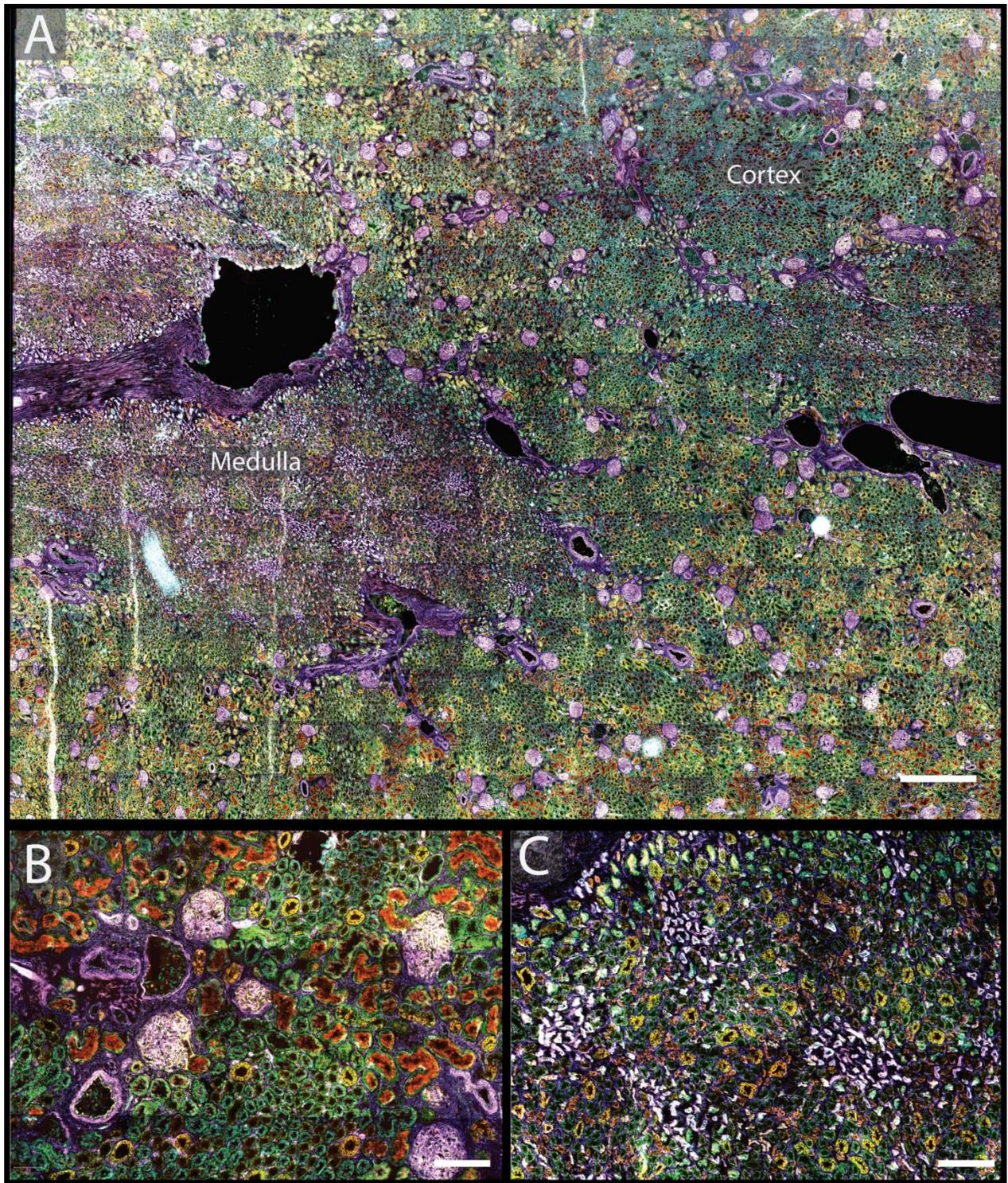
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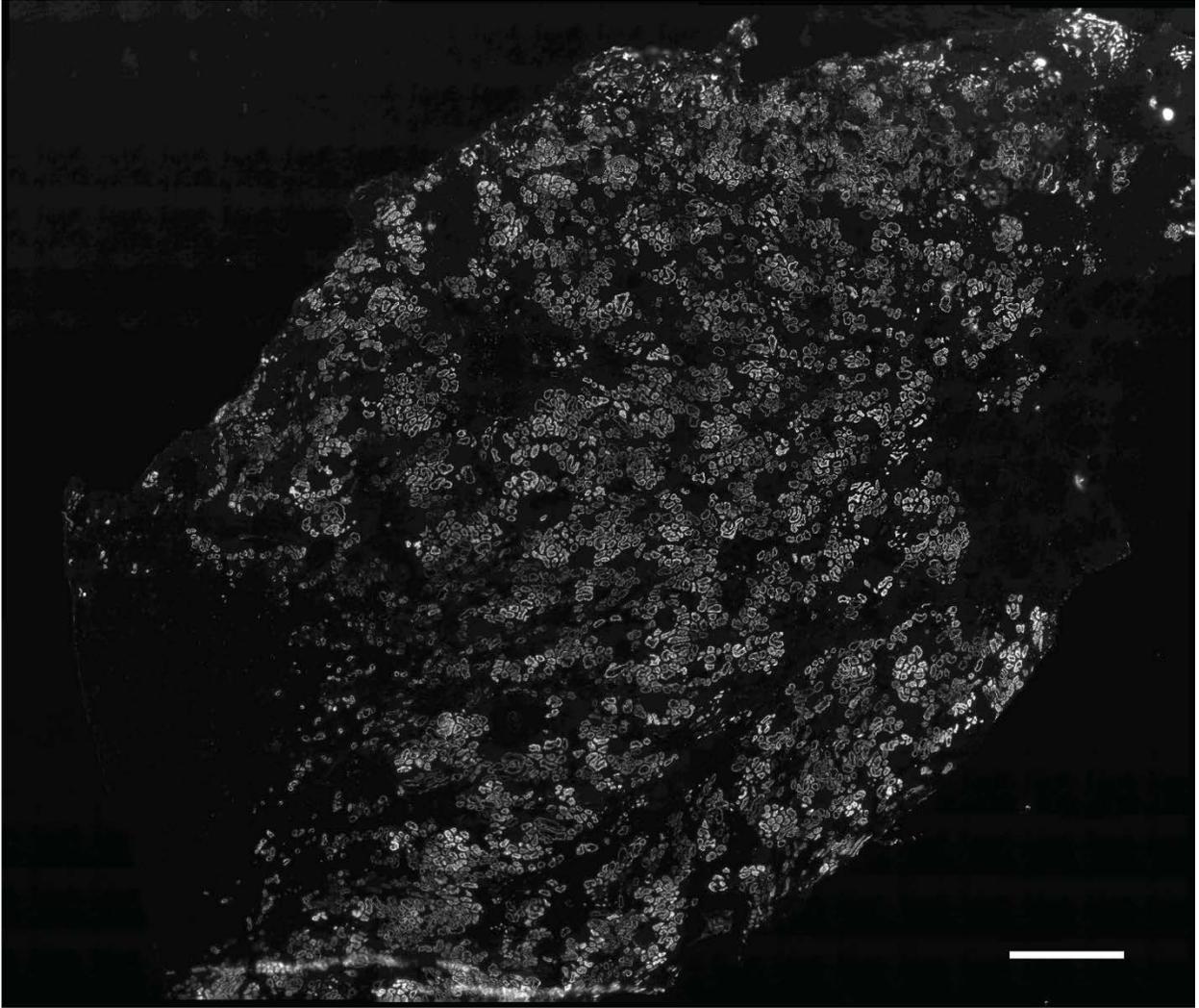
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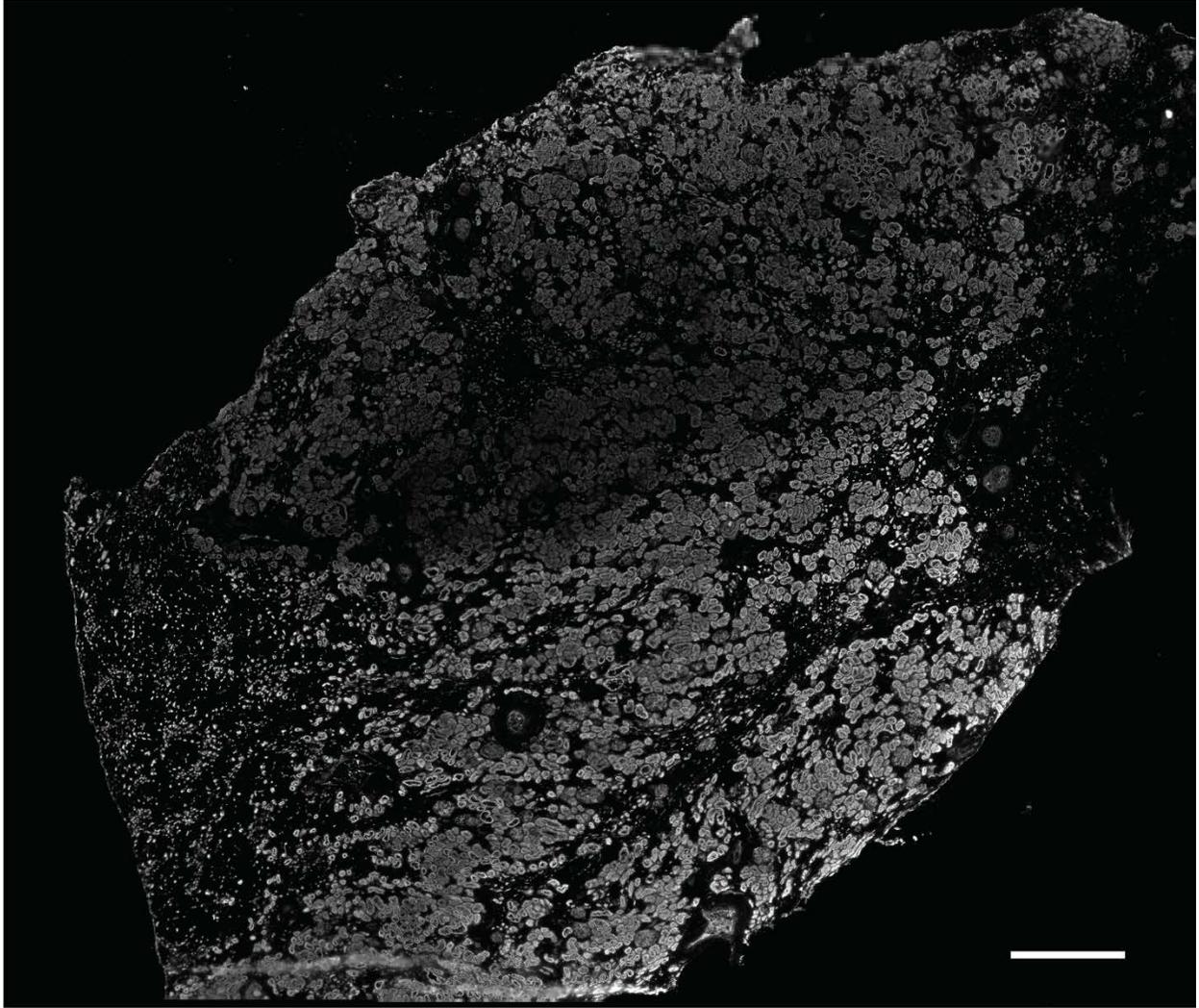
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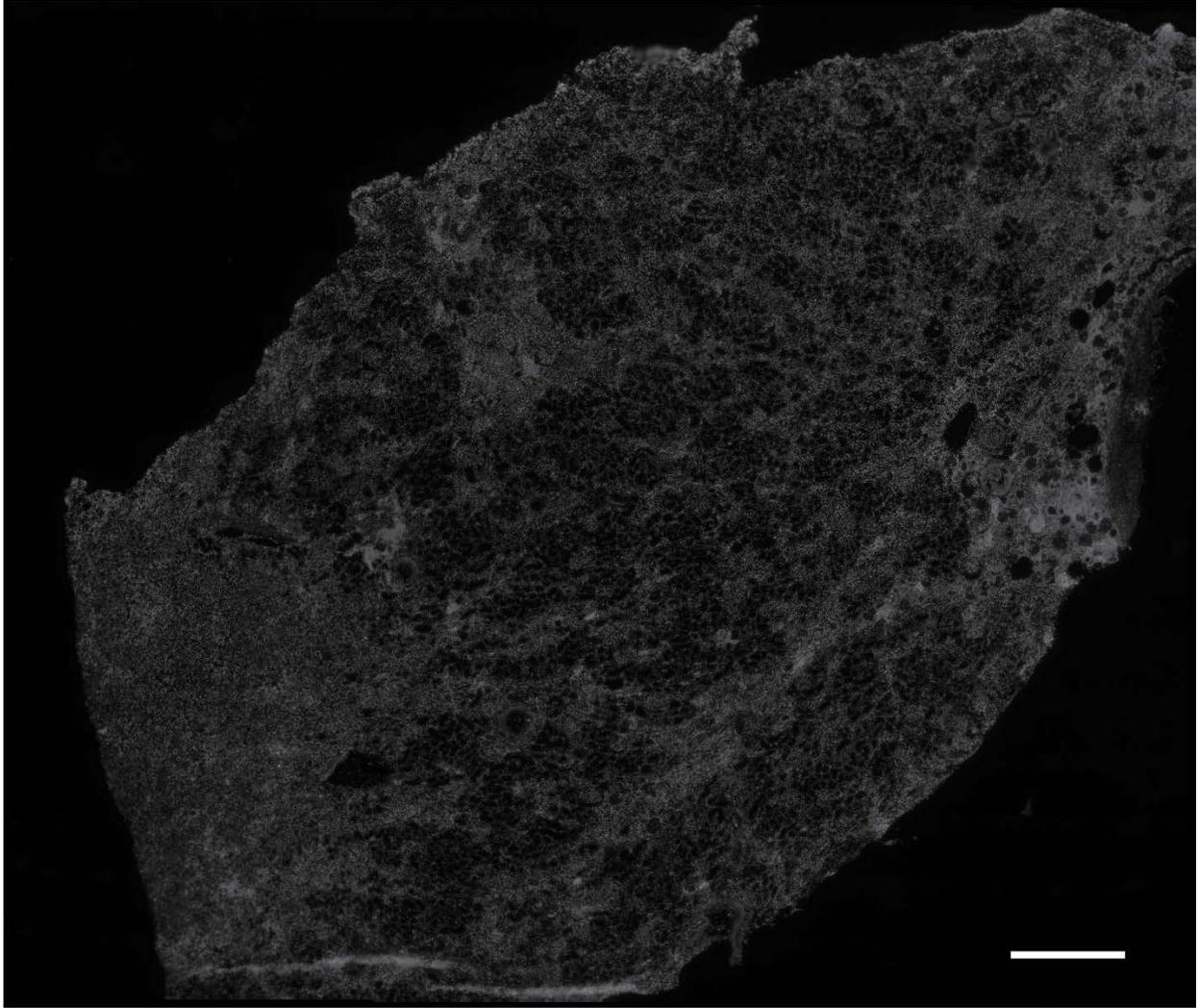
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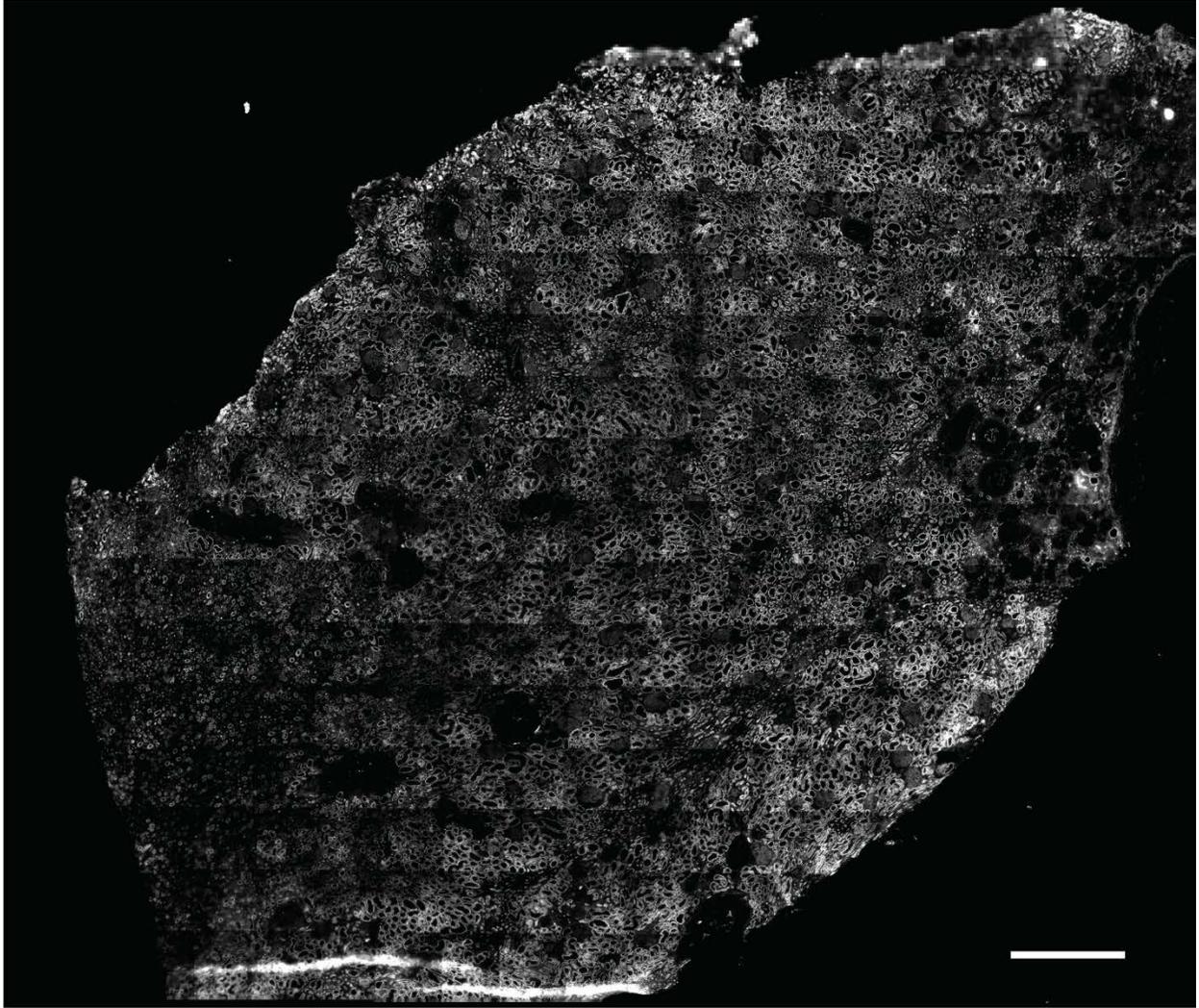
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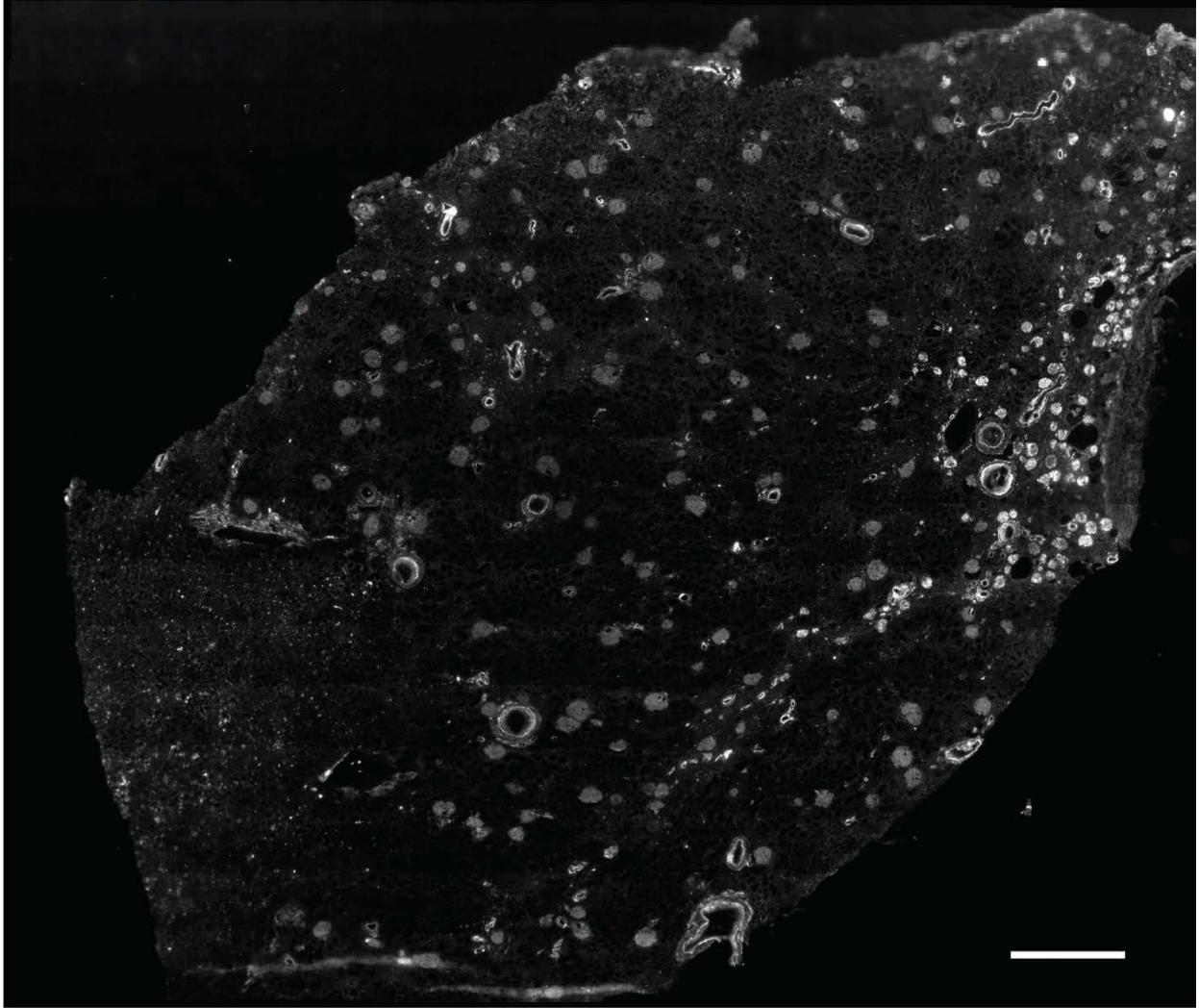
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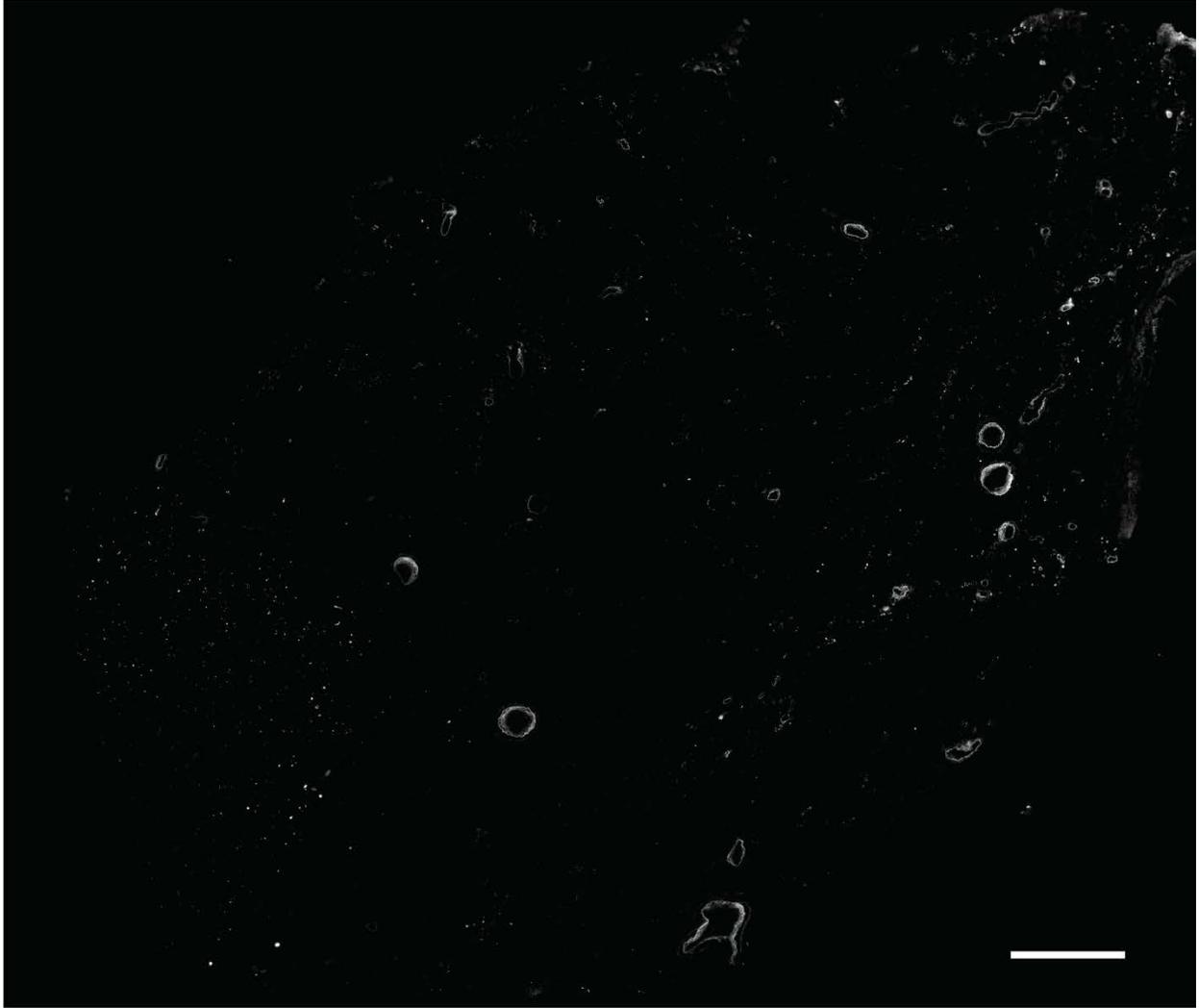
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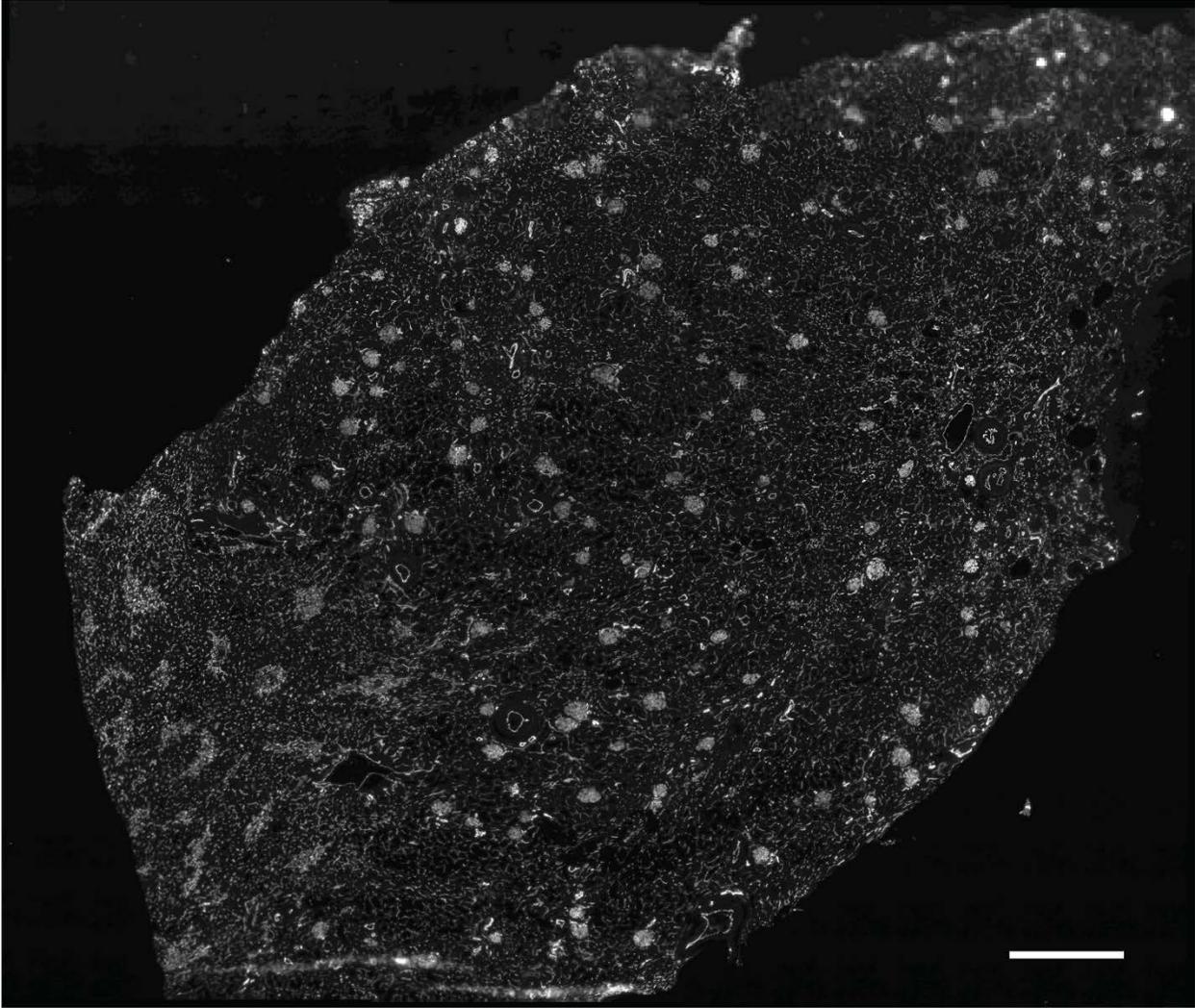
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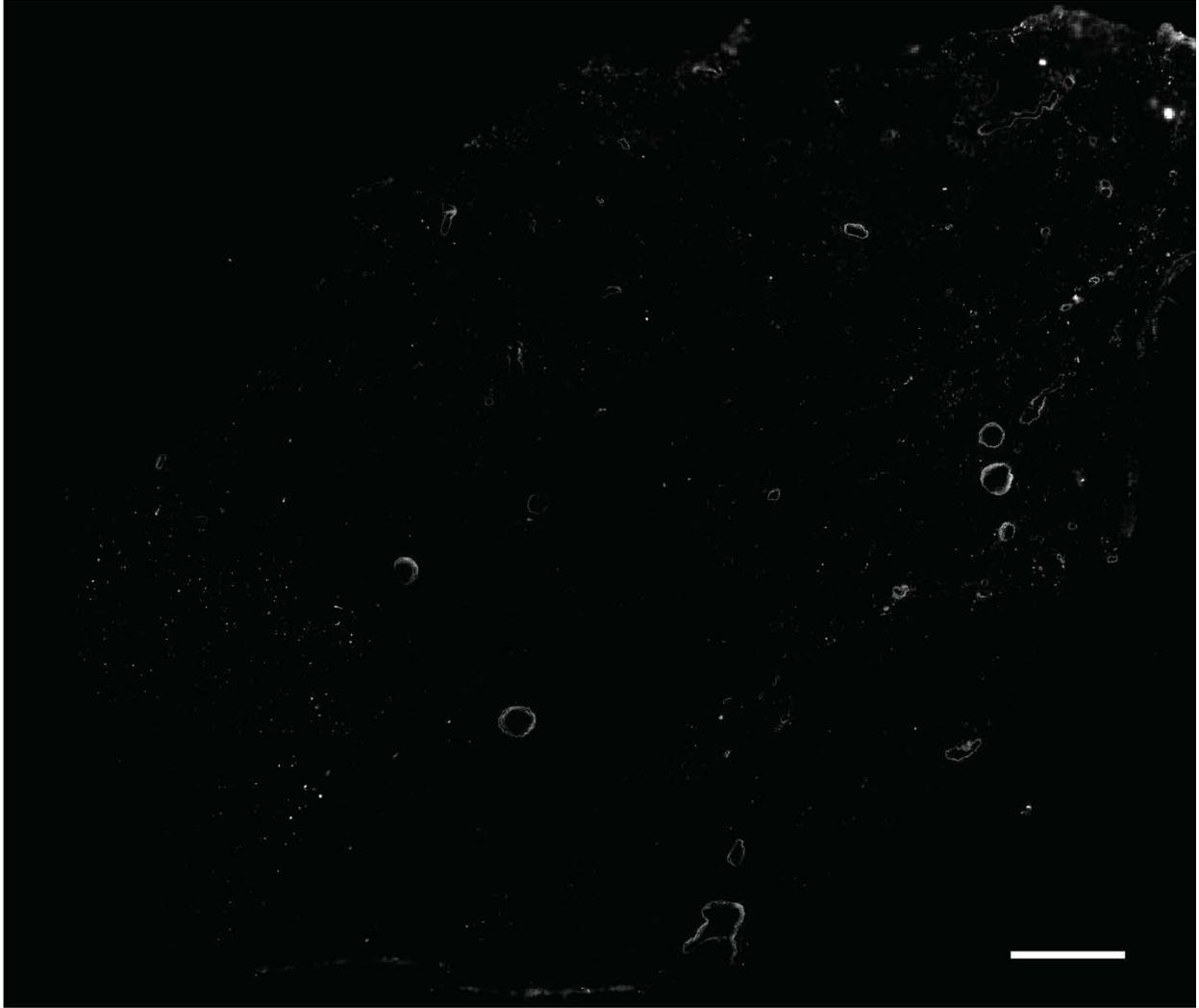
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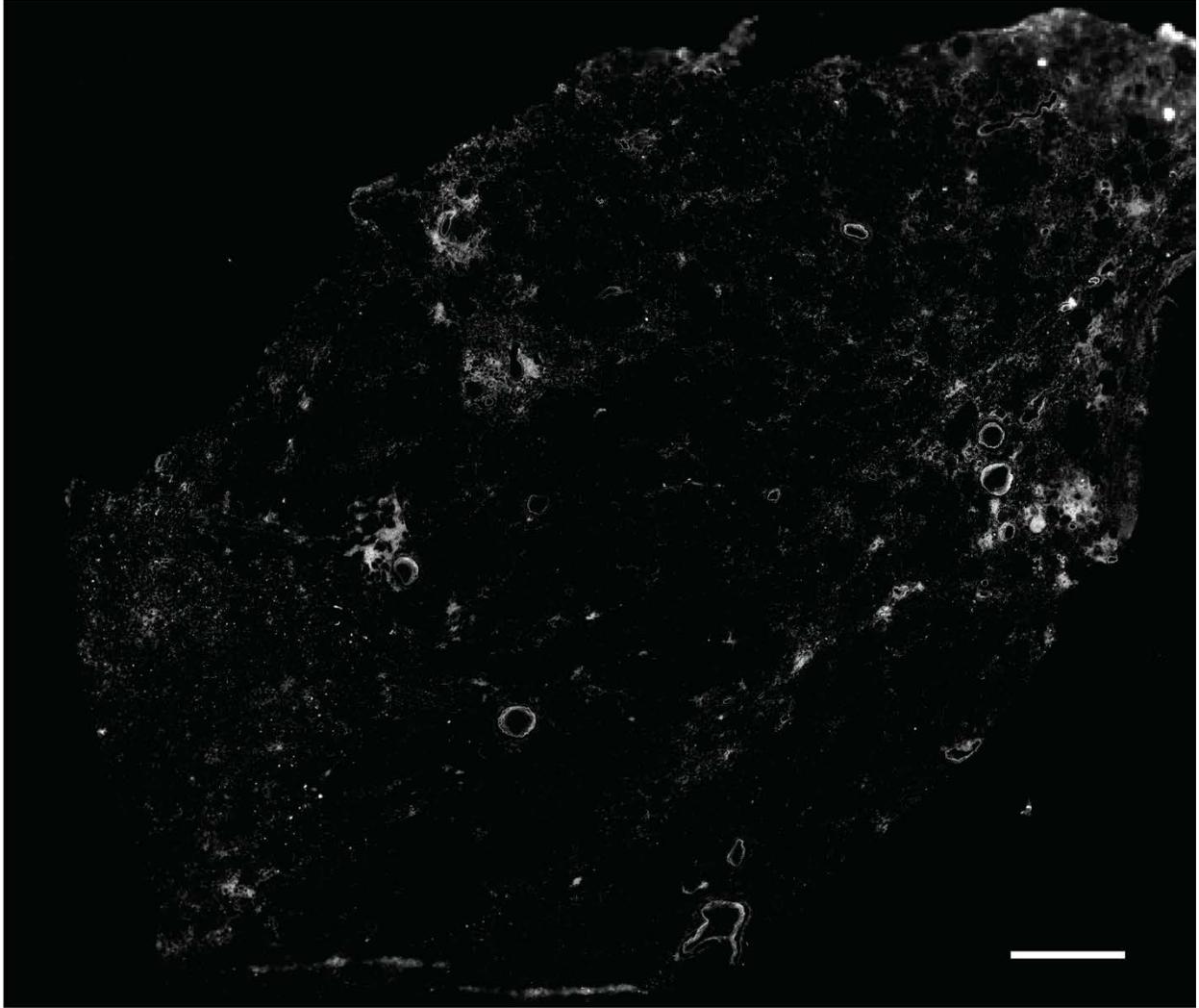
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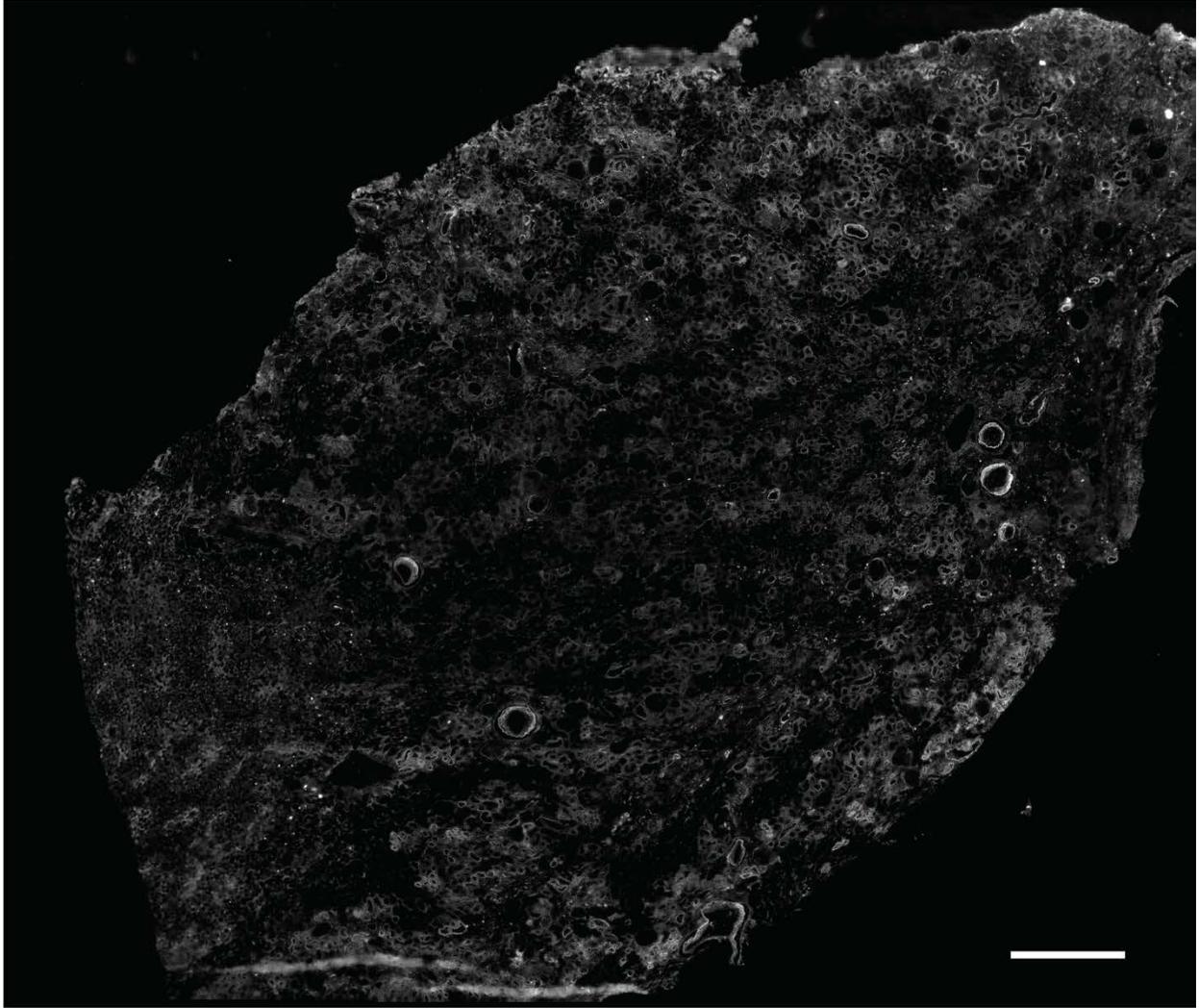
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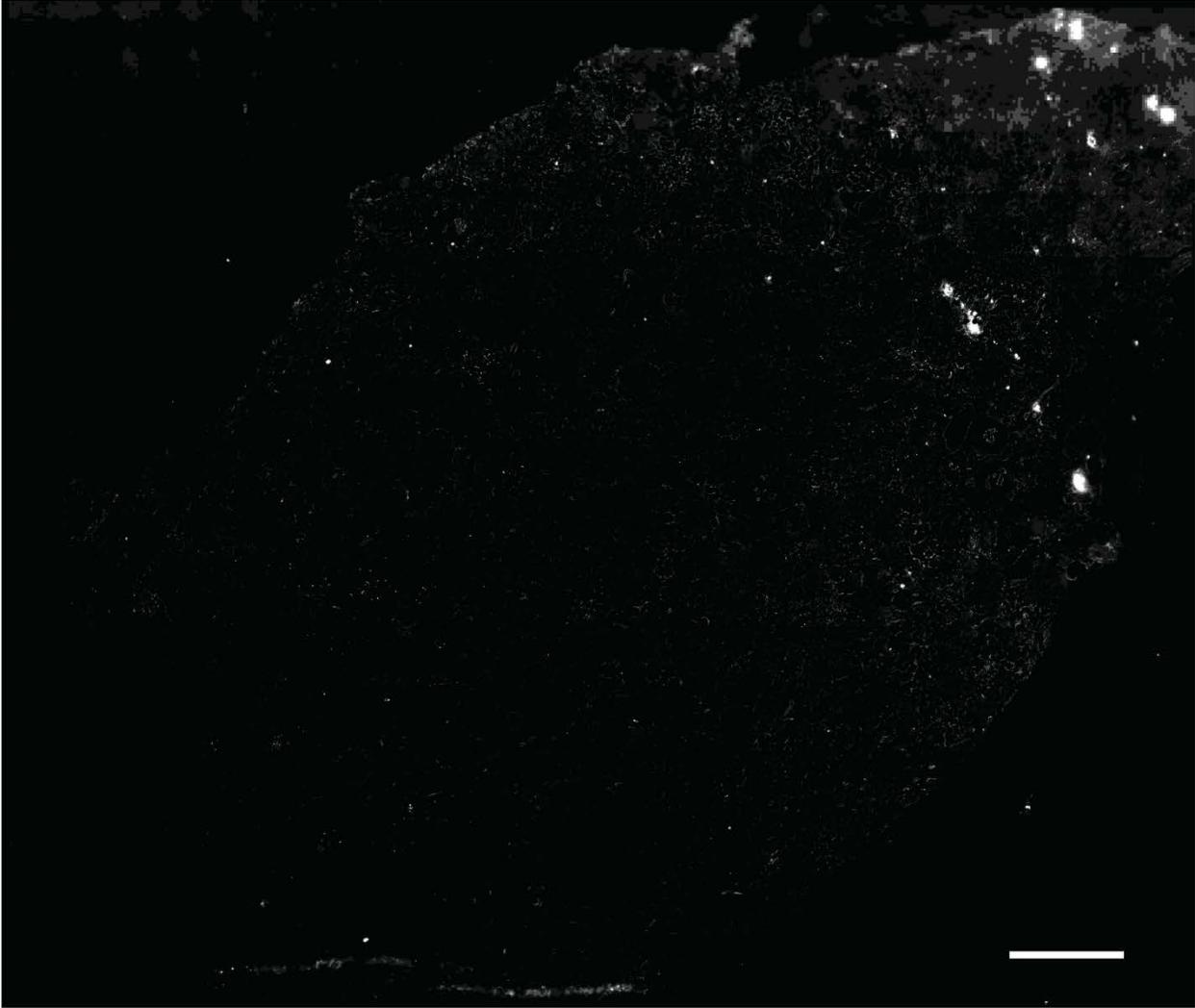
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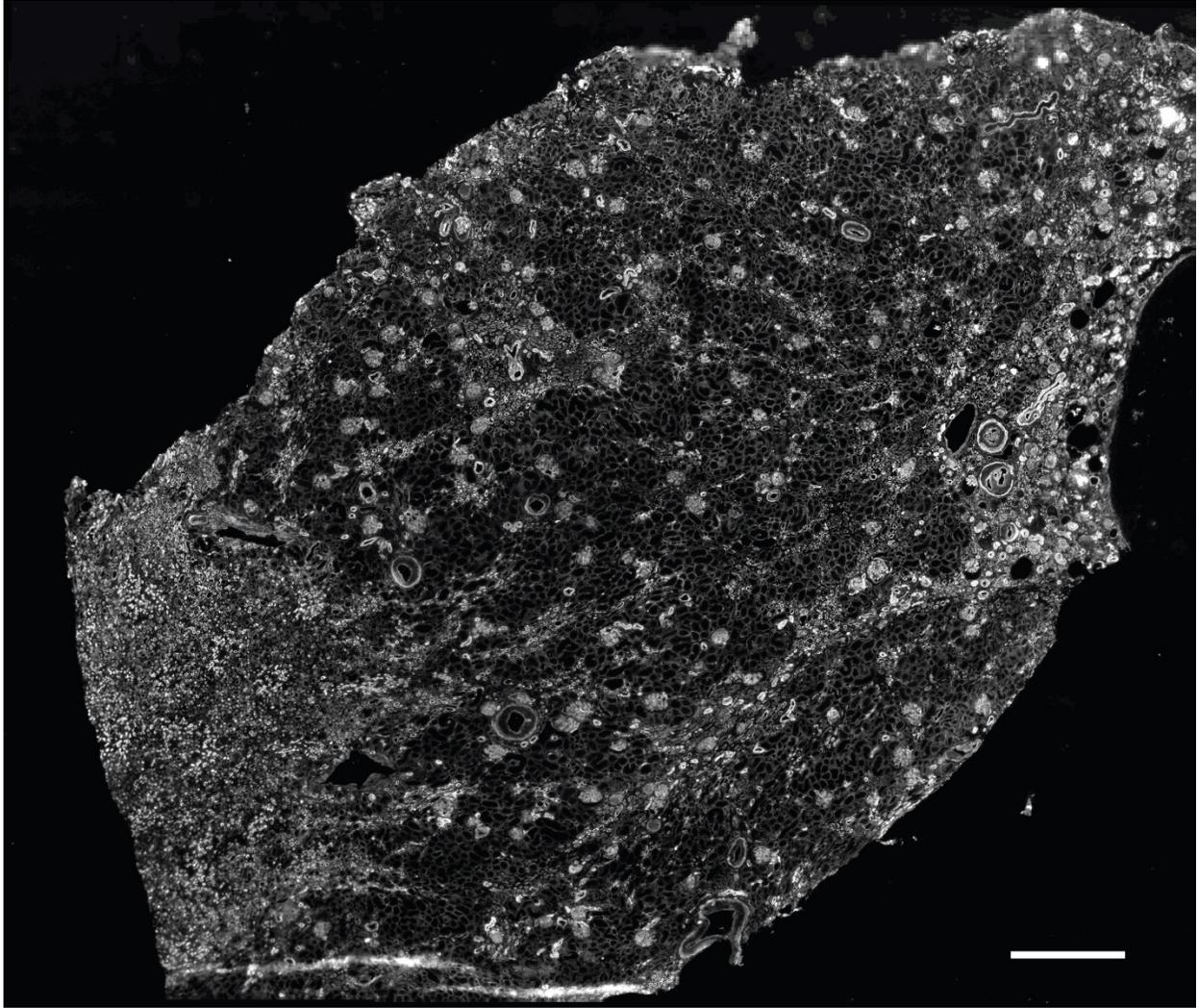
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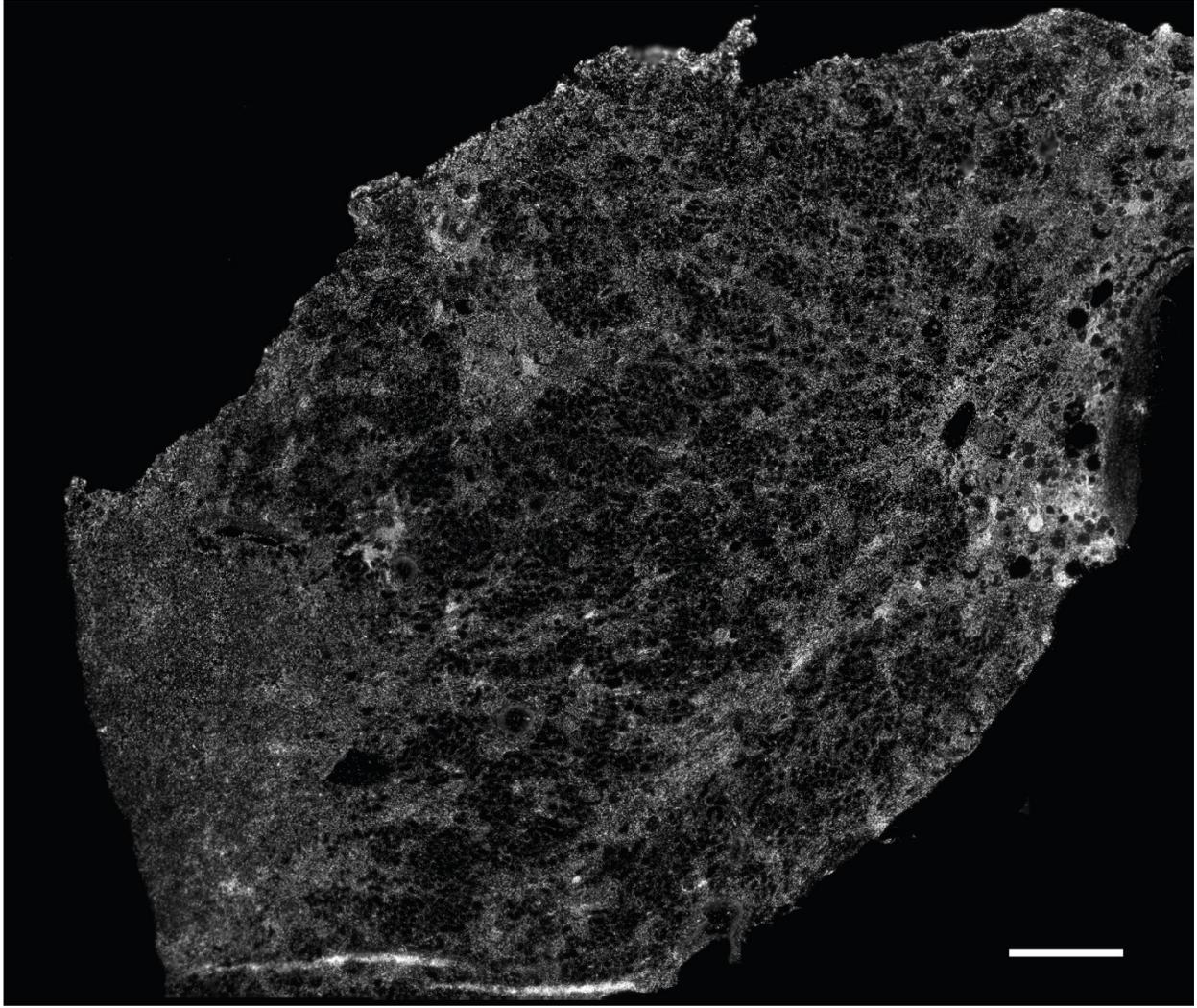
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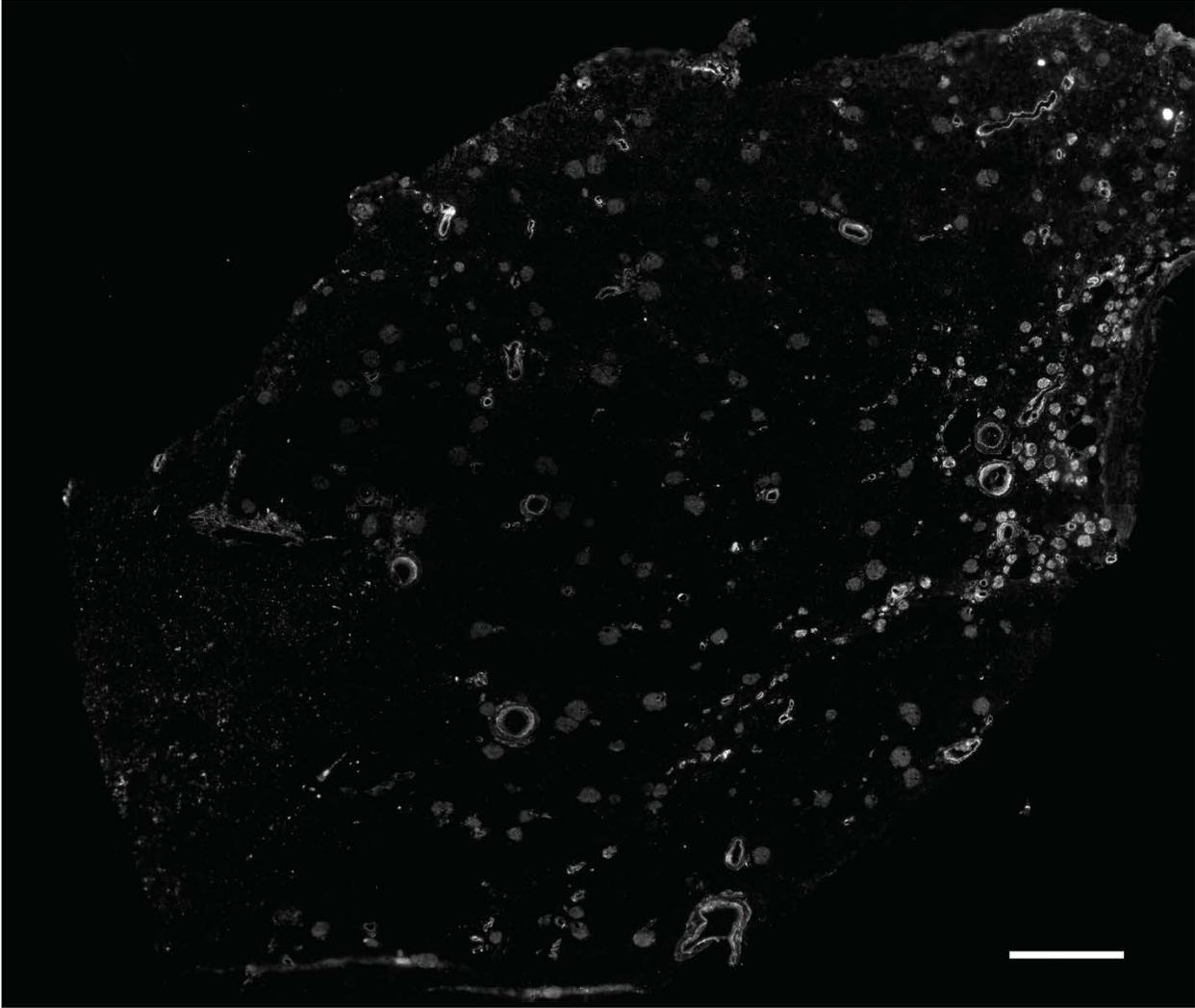
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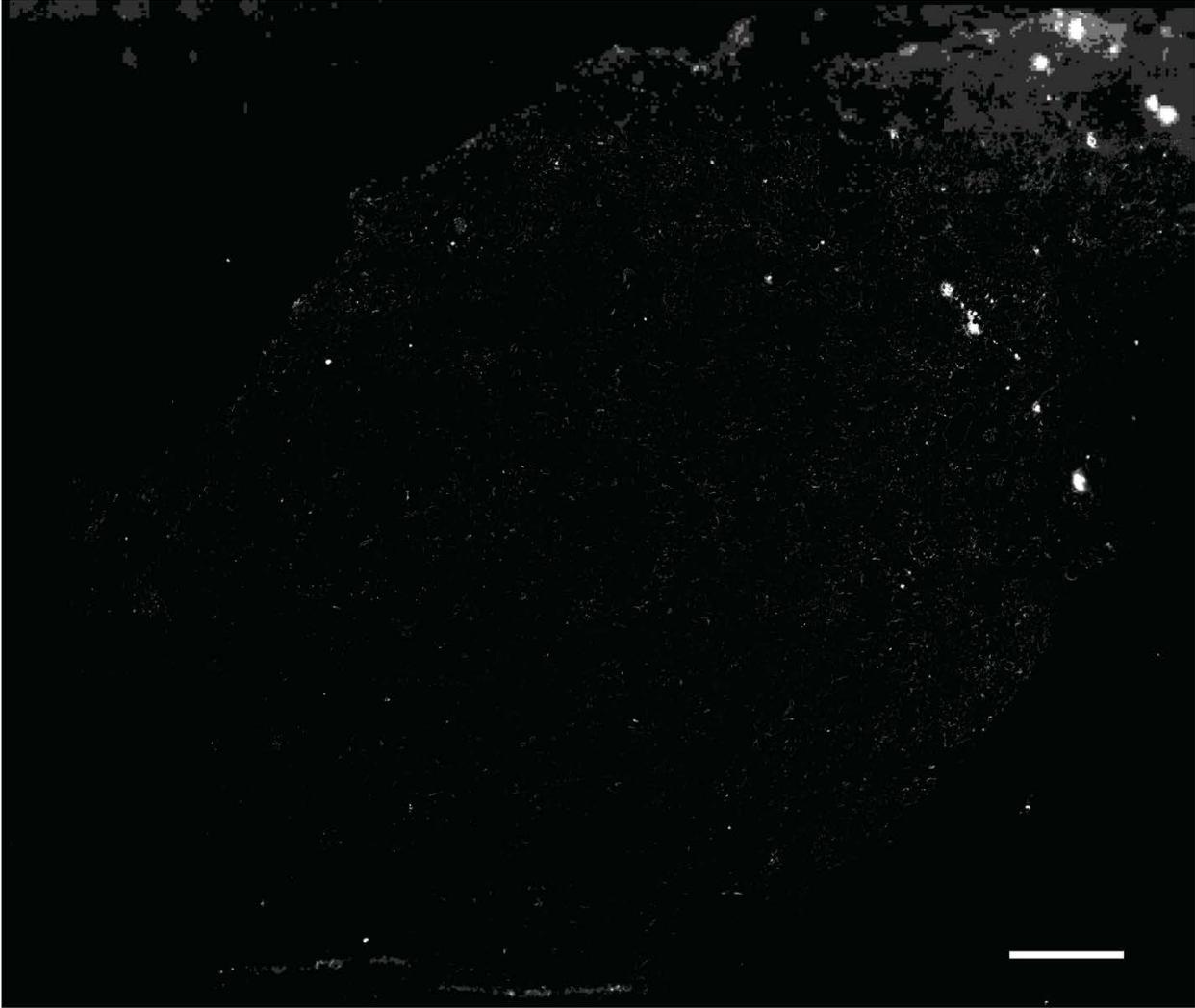
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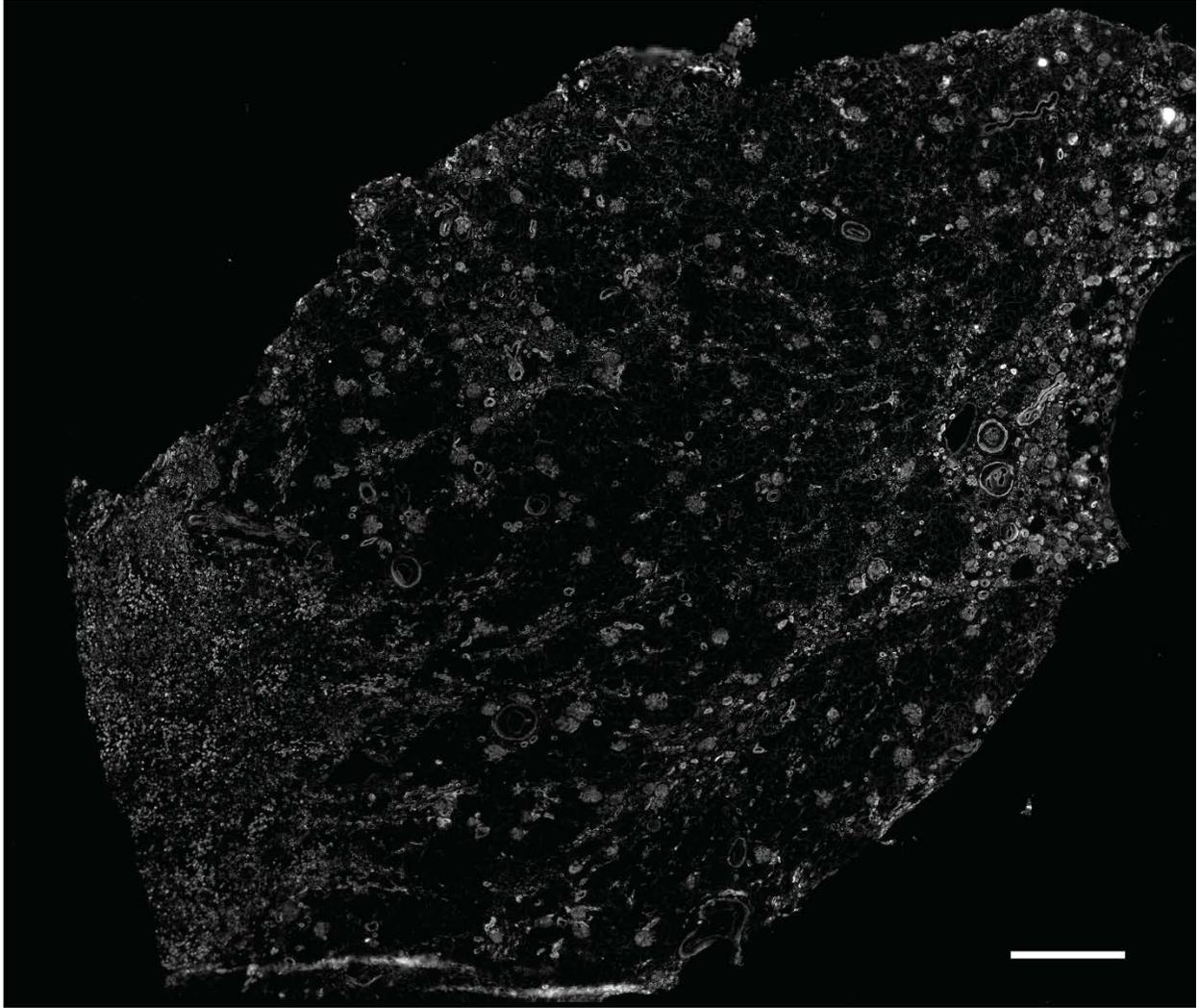
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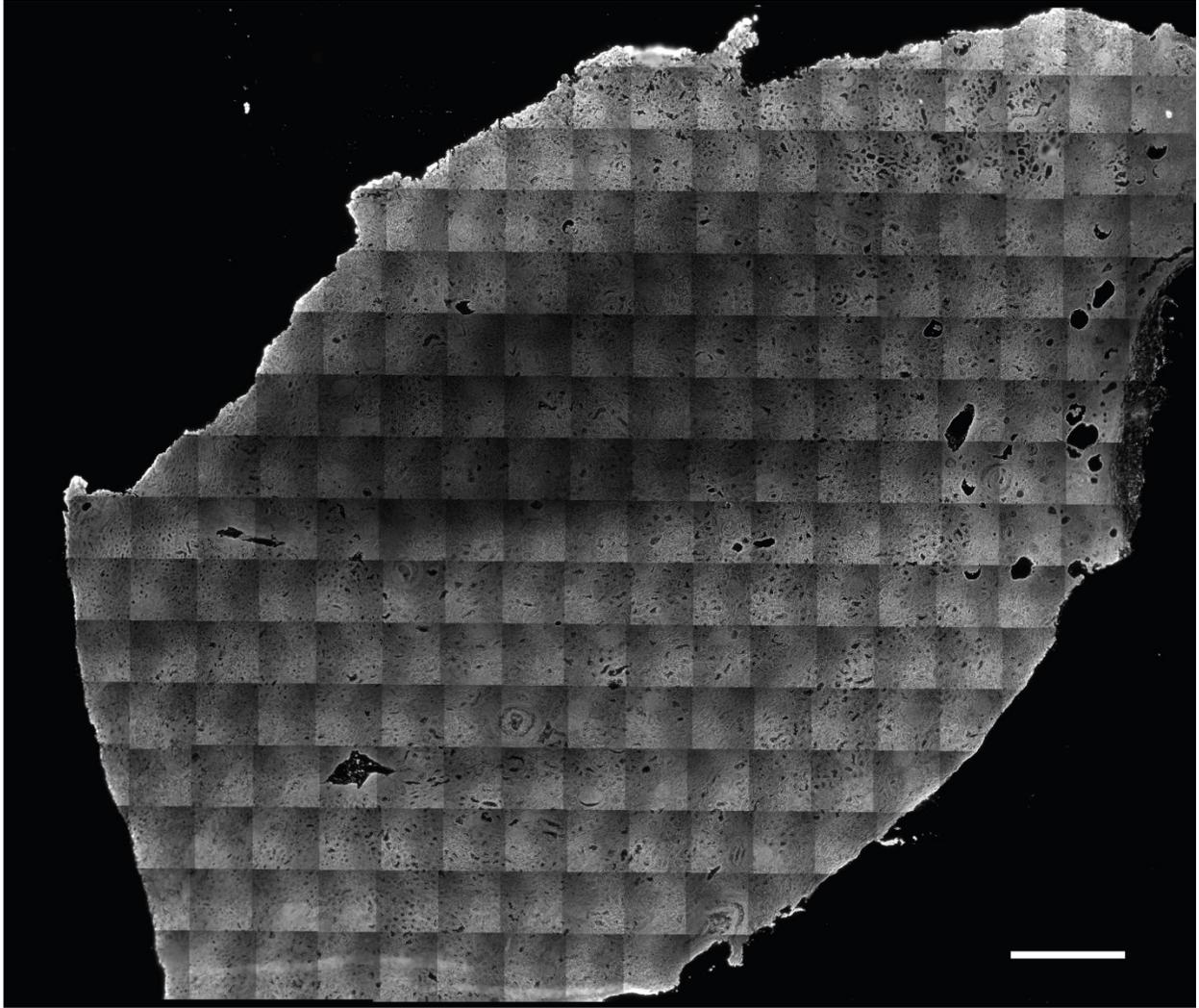
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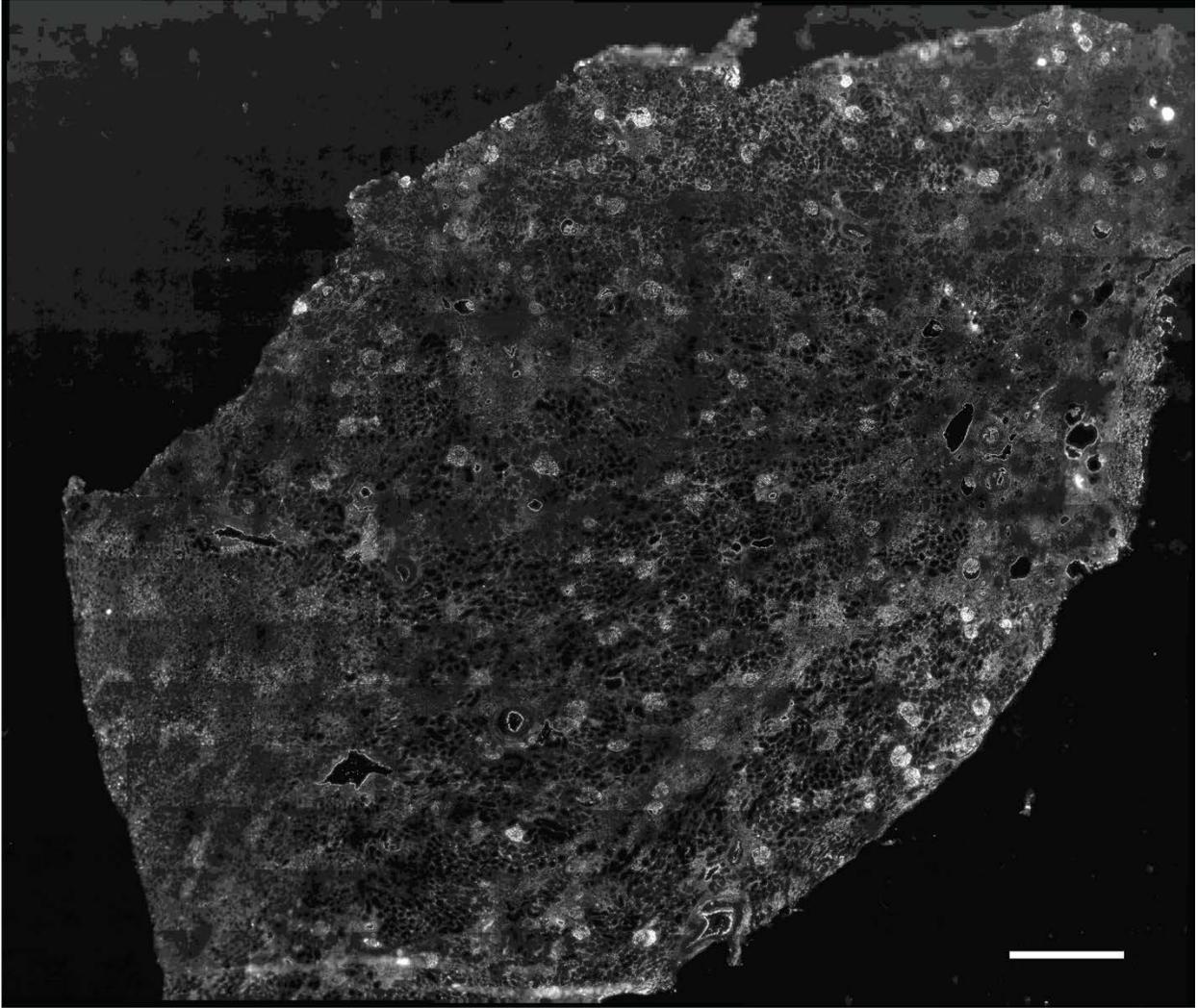
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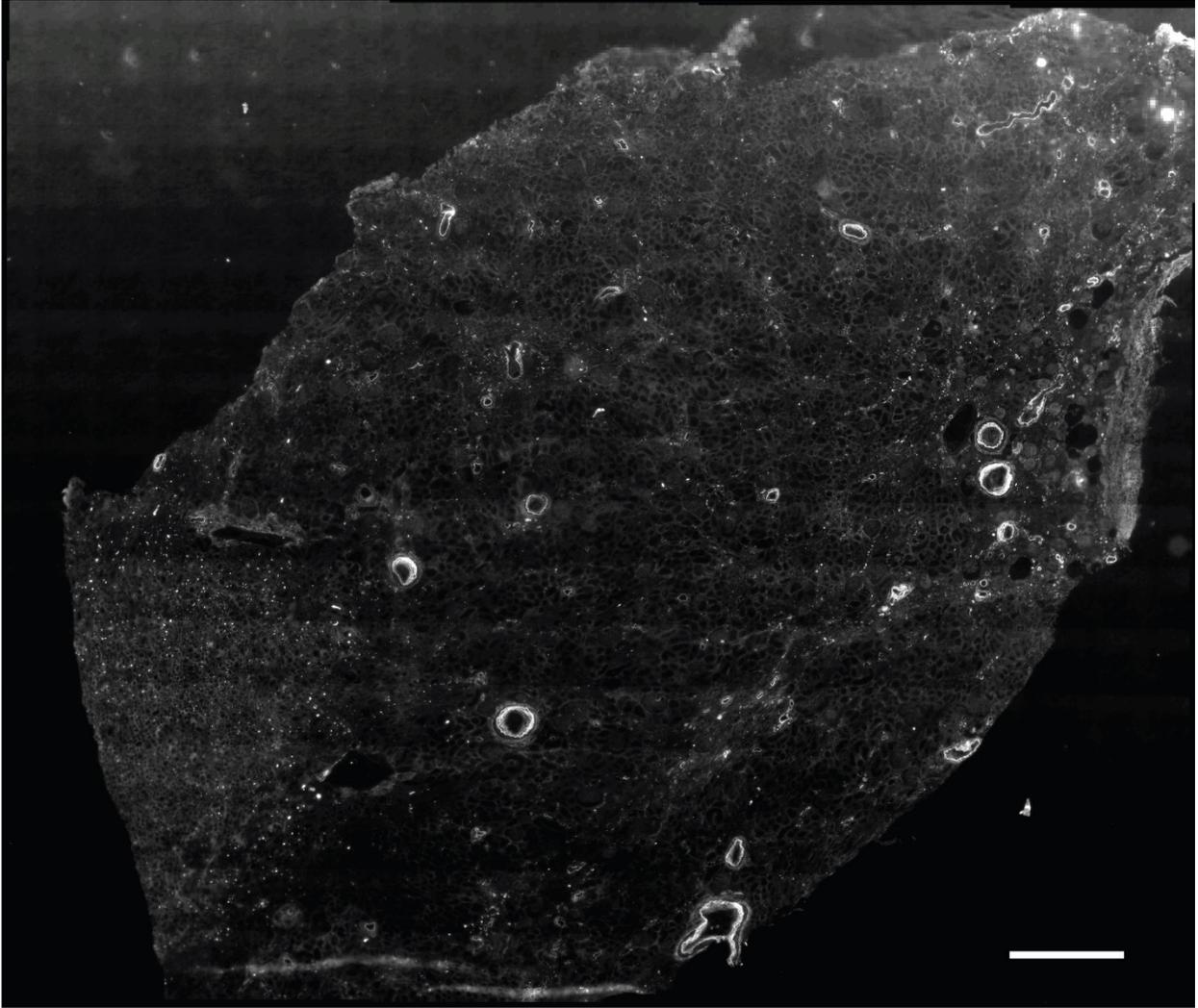
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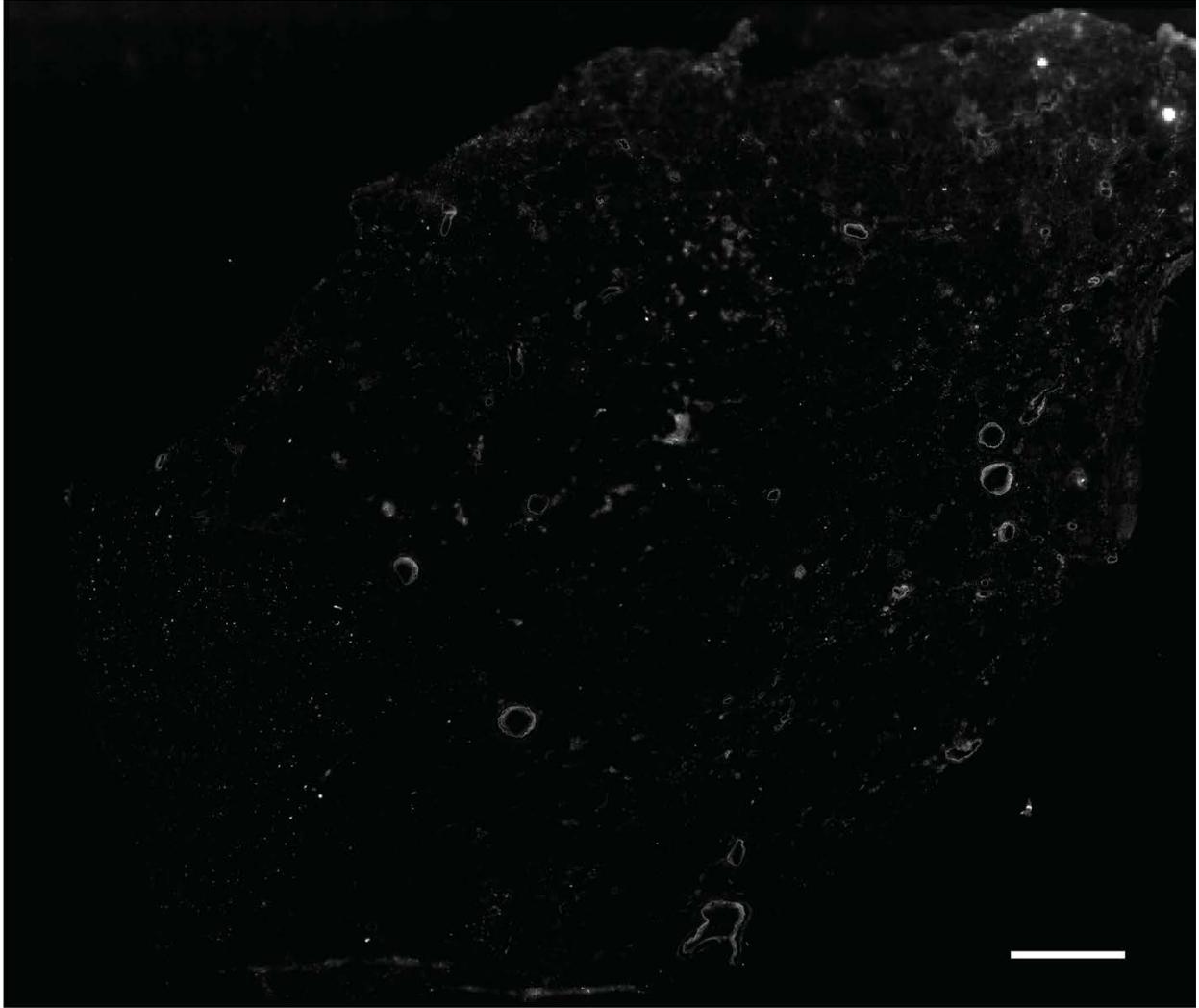
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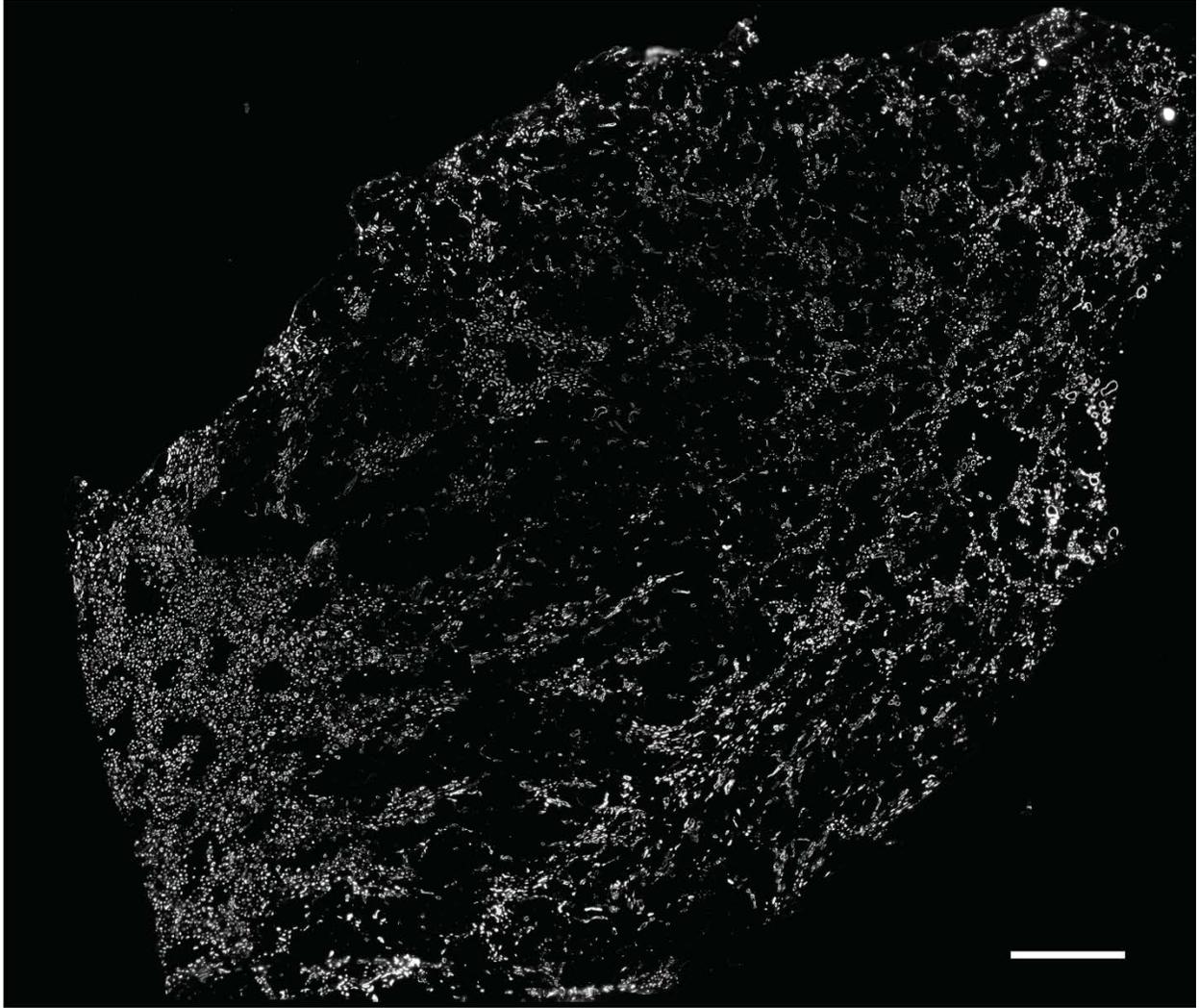
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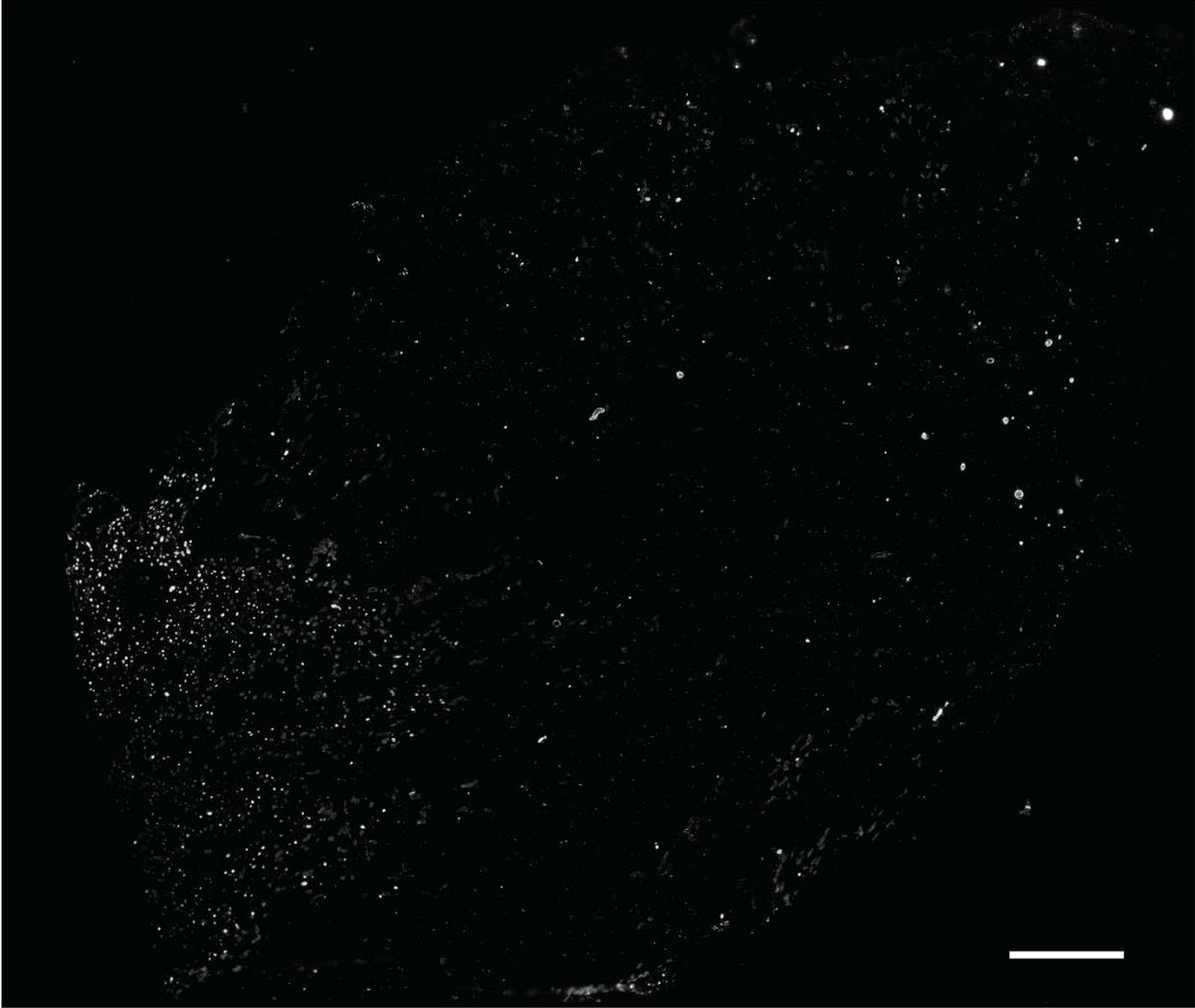
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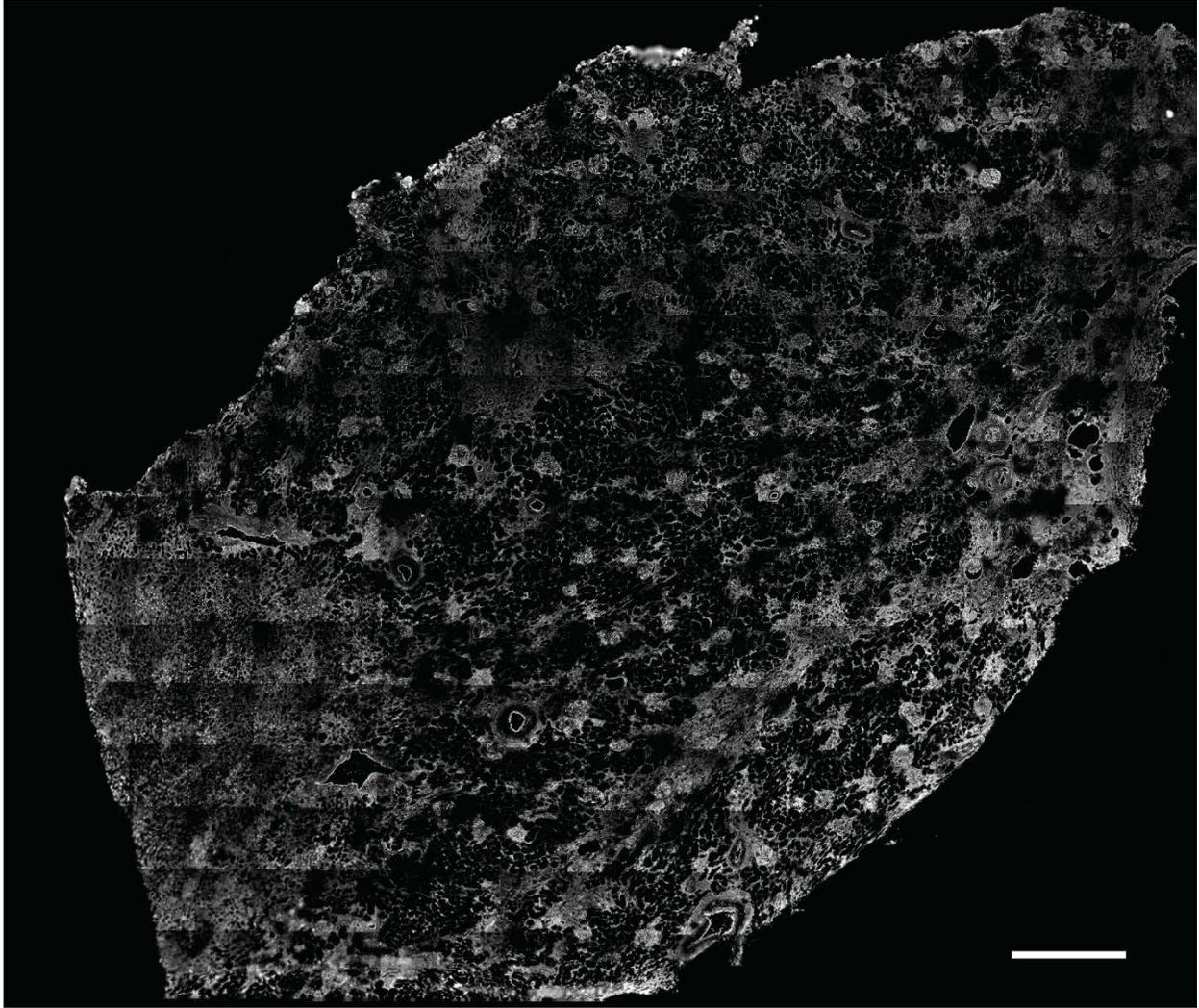
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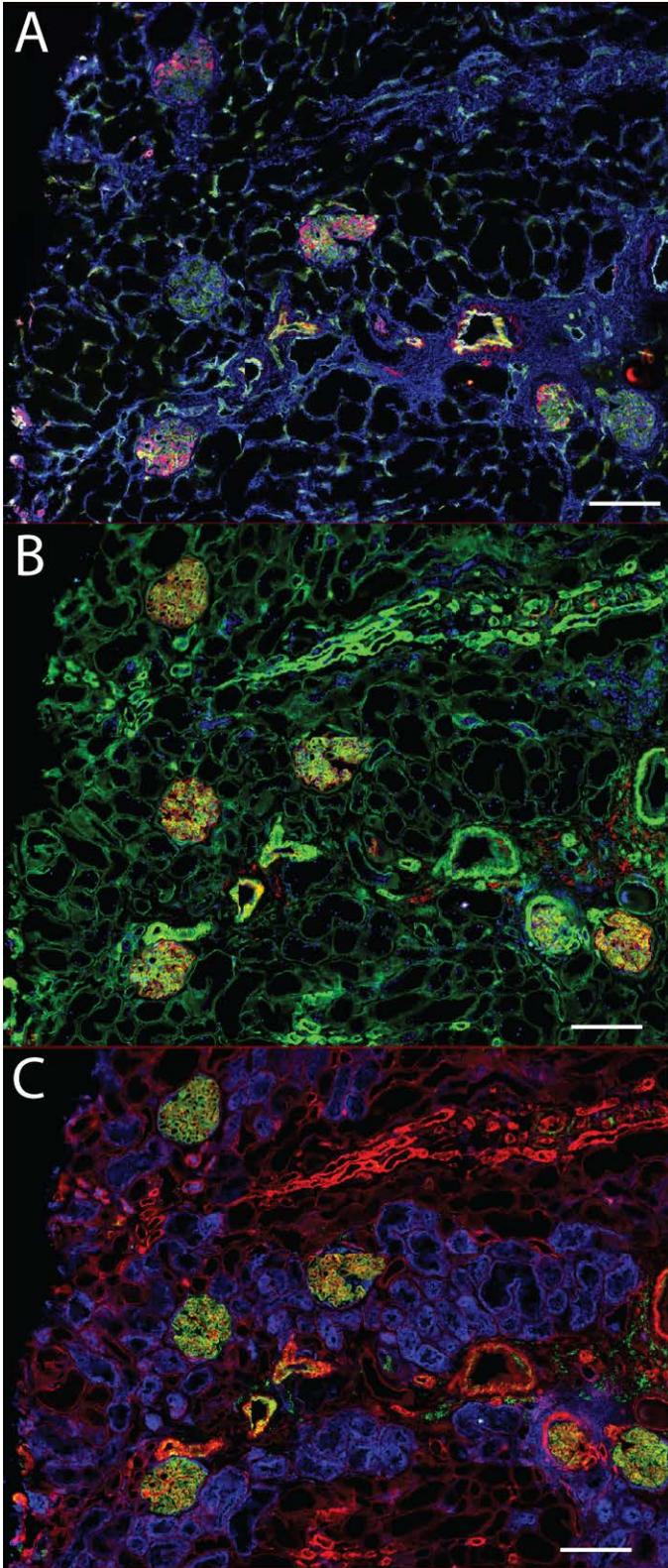
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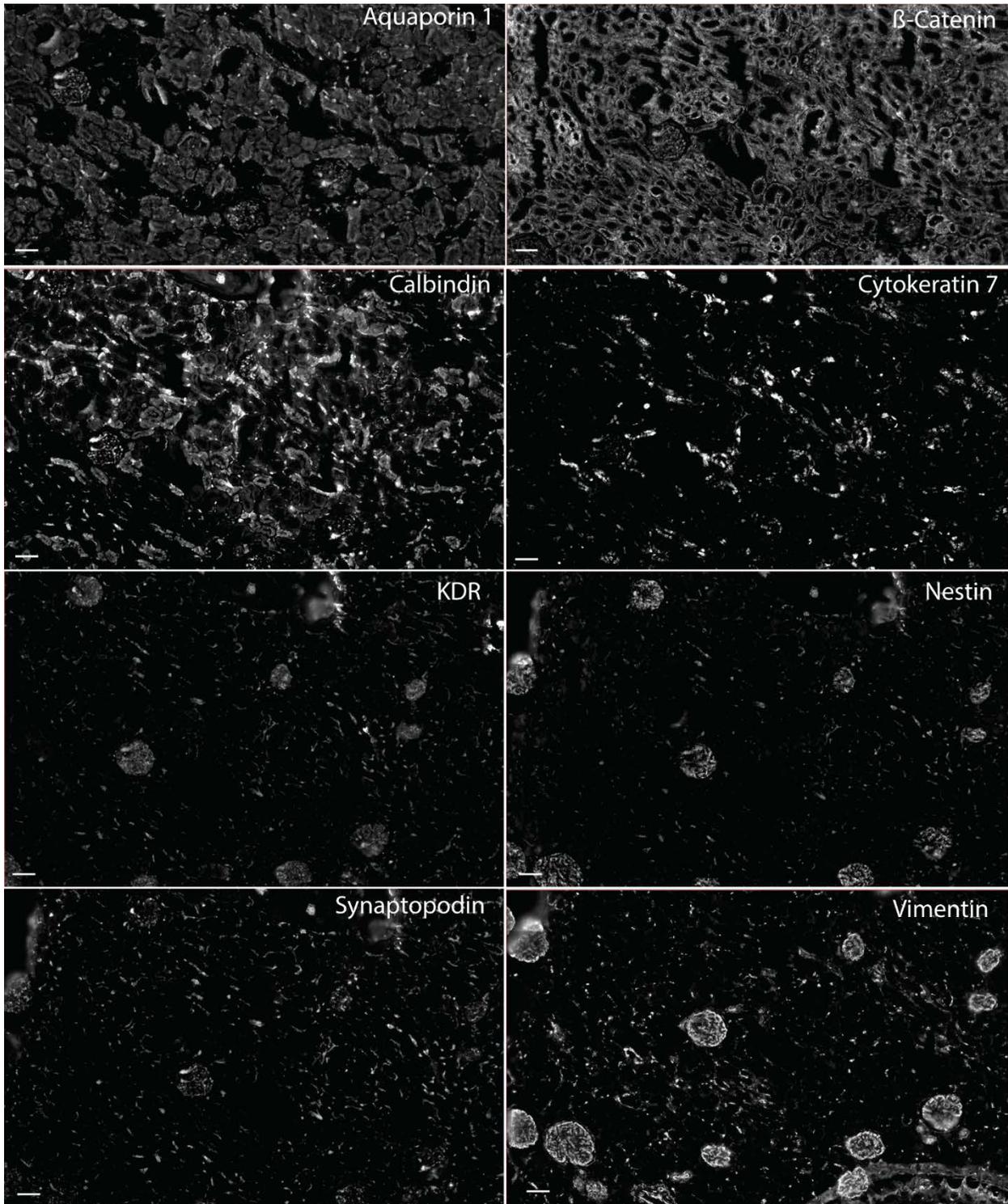
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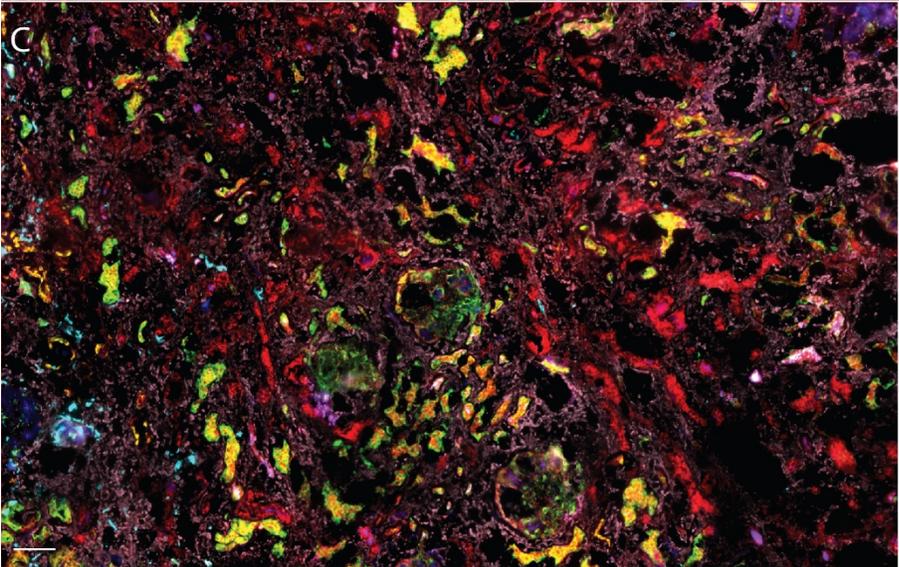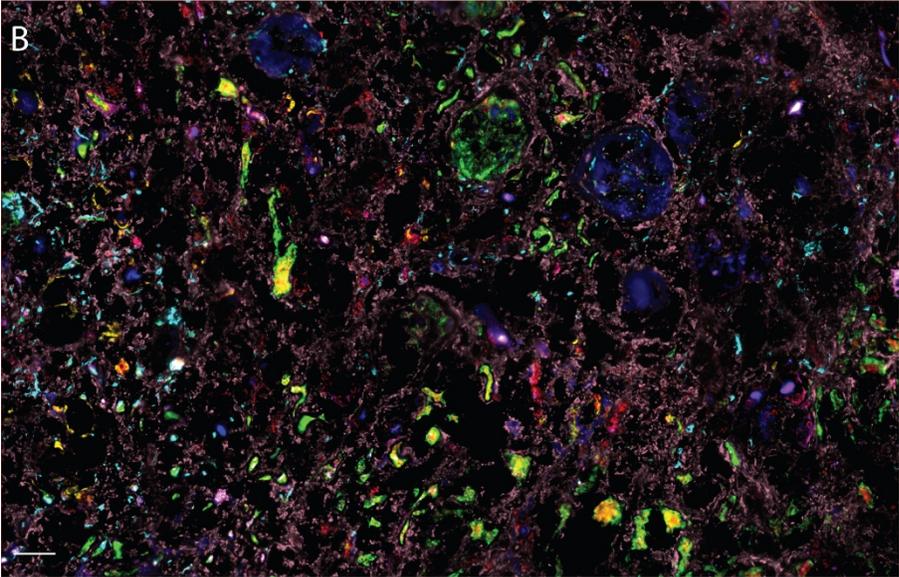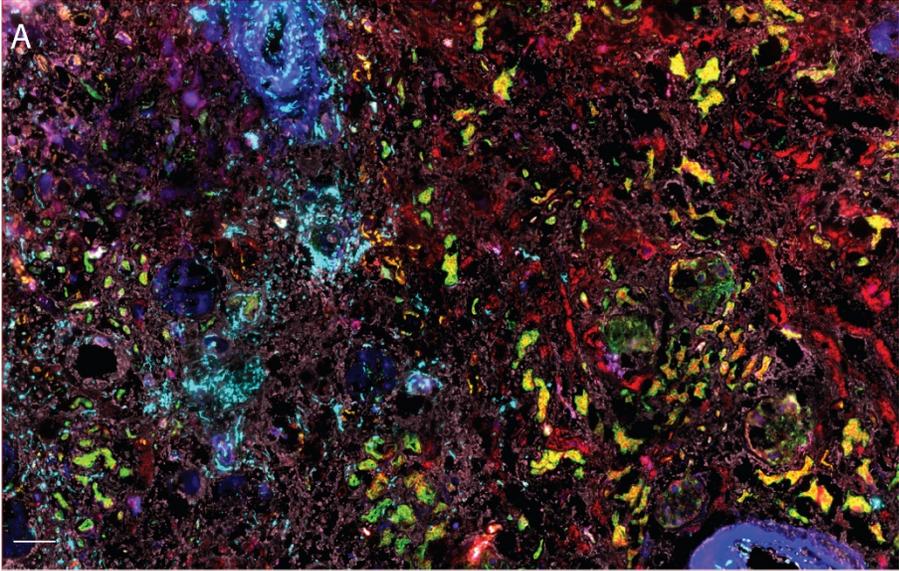
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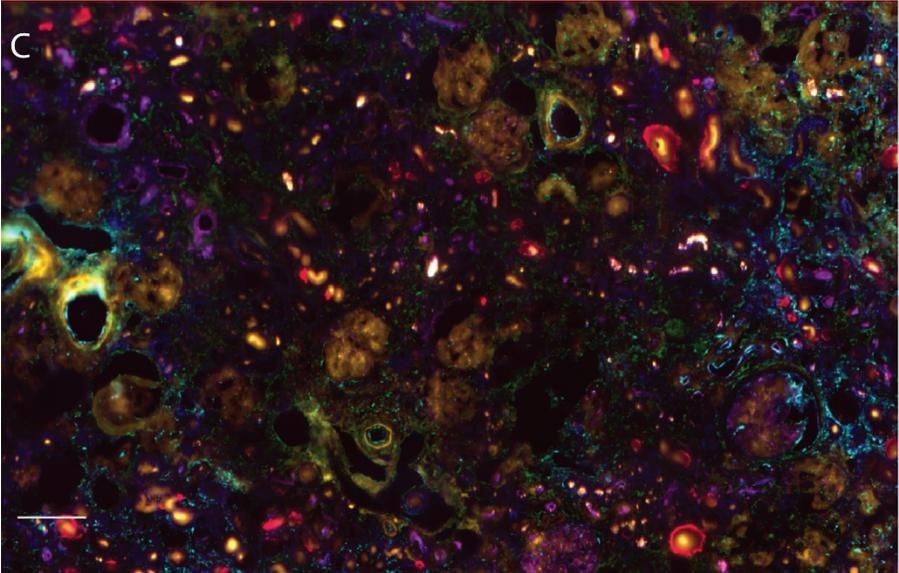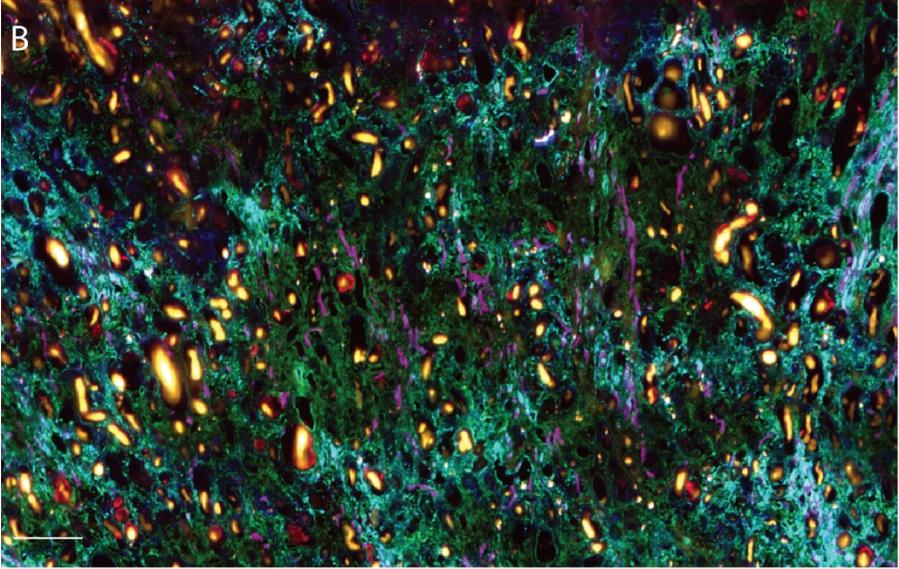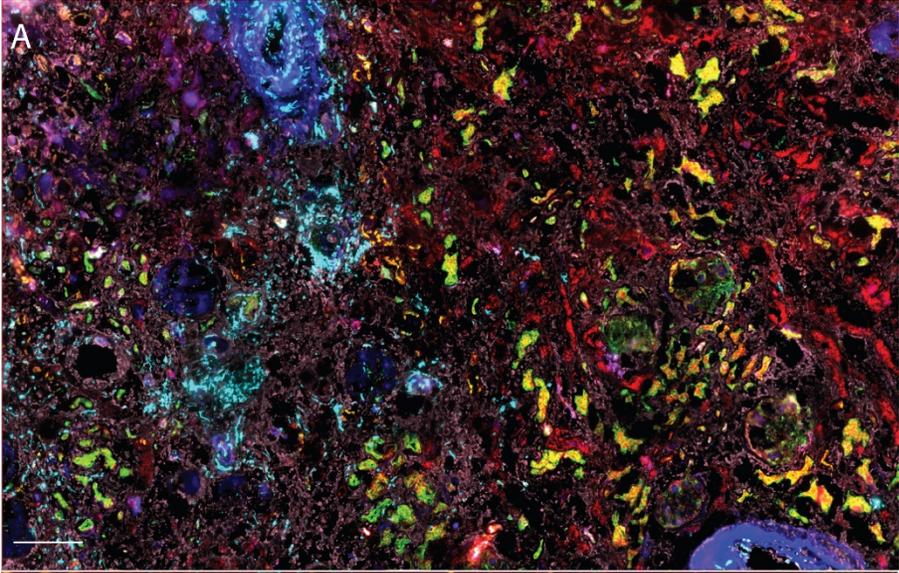
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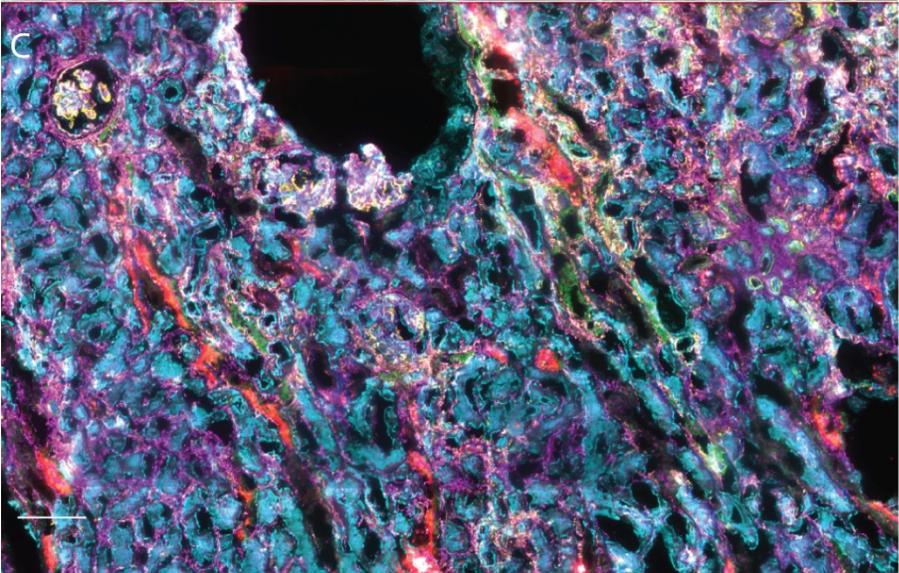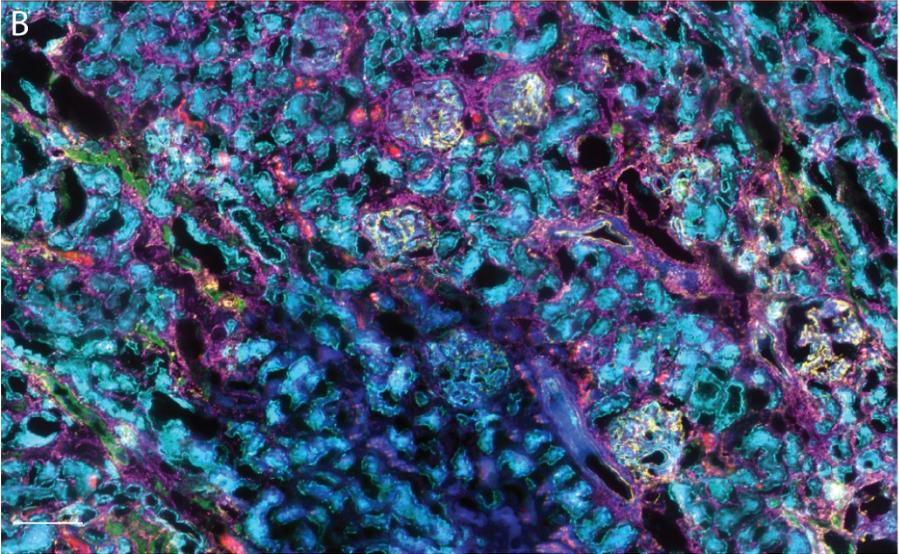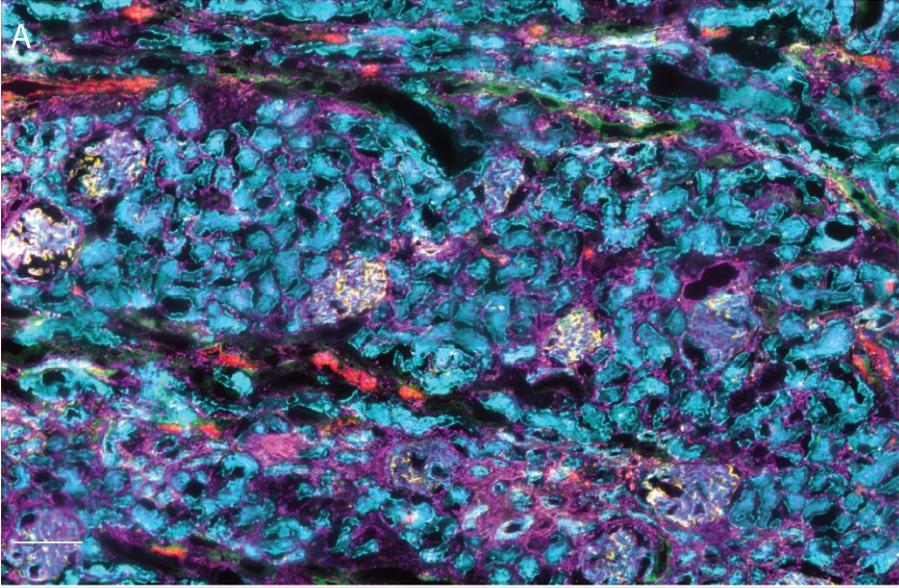
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Extended Methods:

Materials:

HPLC-grade acetone, isopentane, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Sample Preparation:

Human kidney tissue was surgically removed during a full nephrectomy and remnant tissue was processed for research purposes by the Cooperative Human Tissue Network at Vanderbilt University Medical Center. Remnant biospecimens were collected in compliance with the Cooperative Human Tissue Network standard protocols and National Cancer Institute's Best Practices for the procurement of remnant surgical research material. Participants were consented for remnant tissue collection in accordance to institutional IRB policies. The excised tissue was flash frozen over an isopentane-dry ice slurry, embedded in carboxymethylcellulose, and stored at -80 °C until use. Kidney tissues were cryosectioned to a 10 µm thickness and thaw mounted onto poly-L-lysine coated glass cover slips and stored at -80 °C until analyzed. A portion of excised tissue was formalin fixed and paraffin embedded (FFPE) for comparison with fresh frozen tissue. FFPE tissue was sectioned to a 10 µm thickness and mounted onto a poly-L-lysine coated glass cover slip.

Frozen tissues were returned to ~20 °C within a vacuum desiccator before being prepared according to the manufacturer's protocols (Akoya Biosciences, Marlborough, MA). In brief, antigen retrieval was performed using a 5 min incubation in acetone. Tissues were then rehydrated in hydration buffer (Akoya Biosciences) and fixed for 10 minutes in a 1.6% paraformaldehyde (Thermo Fischer Scientific, Waltham, MA) solution diluted in hydration buffer. Tissues were incubated in staining buffer (Akoya Biosciences) for 30 min. Tissues were then incubated with blocking buffer (Akoya Biosciences) and primary antibody cocktail (1:200 antibody dilution) for 3 hr within a humidity chamber at ~20 °C. The samples were rinsed with staining buffer before being fixed in a 1.6% paraformaldehyde solution diluted in storage buffer (Akoya Biosciences) for 10 min. The samples were then rinsed in phosphate buffered saline (PBS), incubated for 5 min in cold methanol (~4 °C), and rinsed again in PBS. Samples were then fixed with fixative solution (Akoya Biosciences) before being stored in storage buffer at ~4 °C until used. It is key to note that all nuclease free water was used to prepare all buffers that were not purchased directly from the manufacturer. Similar protocols were used to prepare FFPE tissue with notable exceptions. Unless otherwise specified, the protocols from the manufacturer were used. In brief, tissue was deparaffinized in xylenes (two, 5 min washes) and graded ethanol (100%, 90%, 70%, 50%, and 30% ethanol in water) for 5 min each wash. Antigen retrieval was performed in a pressure cooking using 0.01 M citrate buffer. Tissue was left under pressure for 20 min and then returned to room temperature before incubation in hydration buffer. The remainder of the procedure is the same as with fresh frozen tissue. While some antibody clones can successfully stain both fresh frozen and FFPE tissue, this is not often the case. Here, eight antibodies successfully could stain both fresh frozen and FFPE tissue (SI Figure 35), although other clones may work on FFPE tissue.

Antibody Conjugation:

Pre-conjugated CODEX antibodies were purchased from Akoya Biosciences. Unconjugated primary antibodies were purchased from Abcam (Cambridge, MA) unless otherwise specified. Purified primary antibodies were purchased without any additives or preservatives, many of which prevent successful

antibody conjugation. Antibodies were conjugated and prepared according to the manufacturer's protocols (Akoya Biosciences) with slight deviations. In brief, 50 kDa molecular weight cut off filters were blocked using 500 μL of a proprietary filter blocking solution (Akoya Biosciences). 50 μg of each antibody were diluted to 100 μL of PBS and filtered. Antibodies were reduced with the reduction mixture (Akoya Biosciences) for a maximum of 25 min at ~20 °C (compared to 30 minutes). The reduction solution was removed by centrifugation (12,000 g) and exchanged with buffer solution (Akoya Biosciences). Each oligonucleotide barcode is rehydrated in 10 μL nuclease free water (Ambion Inc., Austin, TX) and further diluted in 210 μL of conjugation solution (Akoya Biosciences). Respective barcodes are added to the reduced primary antibodies and incubated for 2 hr at ~20 °C. 5 μL of this conjugated antibody solution is removed for later validation. The solution is than purified by buffer exchanging with purification solution (Akoya Biosciences) and stored in storage solution (Akoya Biosciences) at ~4 °C until used. After verifying conjugation, the staining profiles of newly conjugated antibodies were compared to a traditional, indirect immunofluorescence experiment. In brief, half of a primary antibody lot was conjugated to an oligonucleotide barcode and half was kept unconjugated. For comparison, unconjugated primary antibody was visualized using traditional indirect IF with a fluorophore that was spectrally resolved from that attached to the corresponding oligonucleotide barcode conjugated antibody. Both sets of primaries were incubated and visualized concurrently to measure overlap between the conjugated and unconjugated antibodies, demonstrating similar selectivity between the two methods.

As discussed in the main text, most of the tested primary antibodies successfully conjugate to oligonucleotide barcodes. There are still uncertainties surrounding successful antibody conjugation and why some clones conjugate well while others do not, but we preferentially conjugate antibodies that were purified from the manufacturer (e.g. without glycerol, azides, BSA, and other additives), recombinant, extensively validated (e.g. knock out validated), and validated for a variety of systems (e.g. mouse, rat, human) and approaches. While most of these criteria should not affect the conjugation chemistry, the extensive commercial testing and purification lends to better and, perhaps, more robust primary antibodies.

CODEX Multiplexed Immunofluorescence:

The recommended antibody concentrations provided by the manufacturer were used in all cases. Antibodies are diluted in reporter solution (Akoya Biosciences) to a 1:200 dilution in sets of three with the addition of 1 μg of DAPI. The CODEX system automatically dispensed secondary oligonucleotide barcodes automatically. Microscopy was performed on a Zeiss Axio Observer (Carl Zeiss AG, Oberkochen, Germany) using a Colibri 7 LED lightsource (Carl Zeiss) and C13440 camera (Hamamatsu, Shizuoka, Japan). Images were acquired with a 10% tile overlap and a z-stack ranging from 11 to 20 tiles (depending on the tissue size). Images were processed using ZEN (Zeiss). Visualization and cell segmentation was performed using QuPath using the following parameters (8 μm background radius, 1.5 μm sigma, 10-100 μm^2 allotted nuclear area, 150 intensity threshold, and 10 μm cell expansion.[S1] Signal to background was determined using the initial autofluorescence image as background/noise, so positive signal must be greater than 10 times background signal. Neighborhood analysis was performed using MATLAB and CytoMAP.[S2] In brief, neighborhoods were established 40 μm from the cell center. Signal was normalized to the max mean fluorescence intensity values and the number of clusters was determined using the Davies Bauldin criteria. Regions were determined using default neural network criteria.

S1.   Bankhead P, Loughrey MB, Fernández JA, et al. Q**uPath: Open source software for digital pathology image analysis**. *Scientific Reports.* 2017;**7**:16878.

S2.   Stoltzfus CR, Filipek J, Gern BH, et al. **CytoMAP: A Spatial Analysis Toolbox Reveals Features of Myeloid Cell Organization in Lymphoid Tissues**. *Cell Reports.* 2020;**31**:107523.