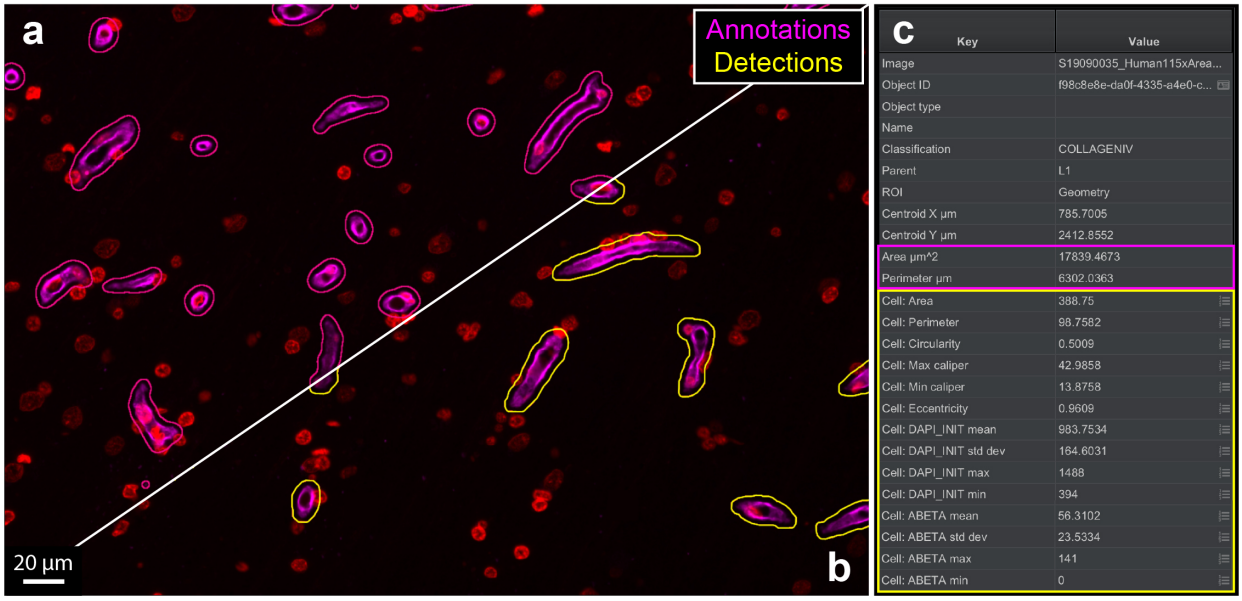
Title: *AnnoTools*: A modular object-based framework for multiplexed immunofluorescence image analysis

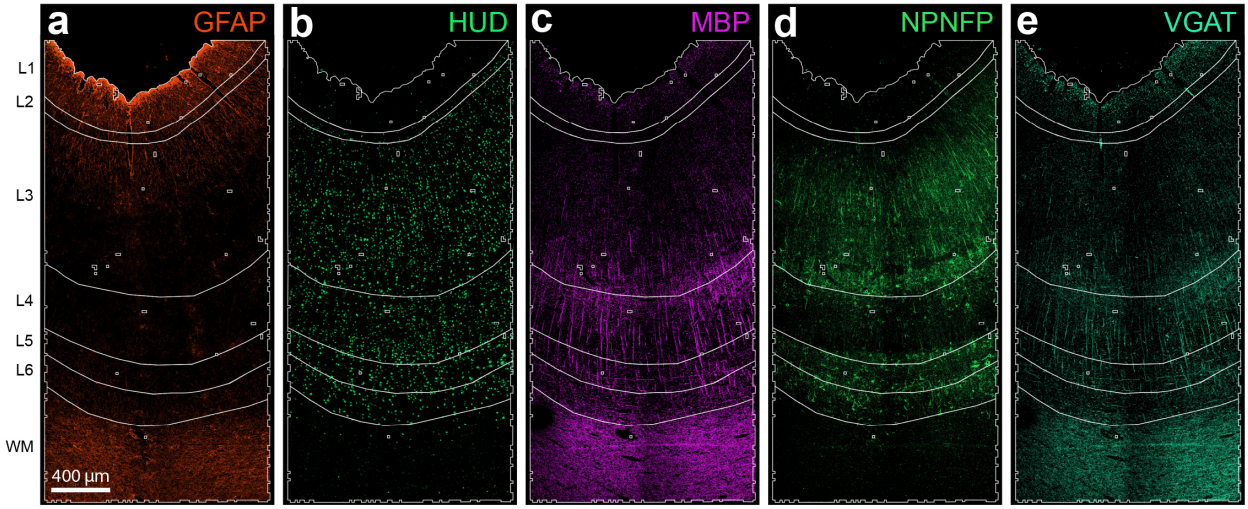
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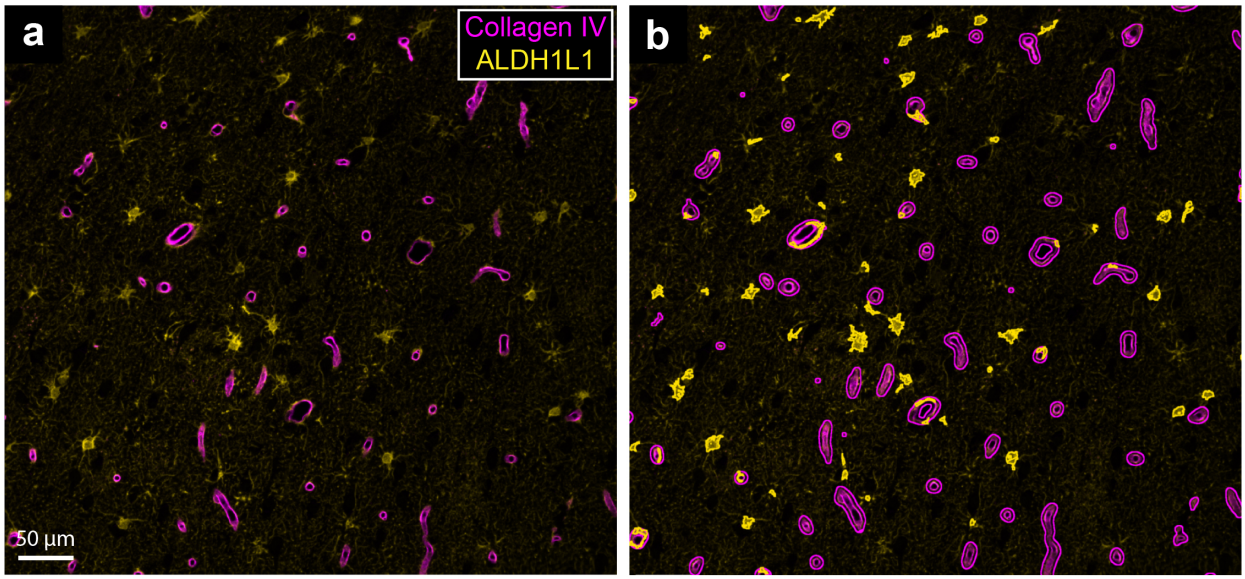
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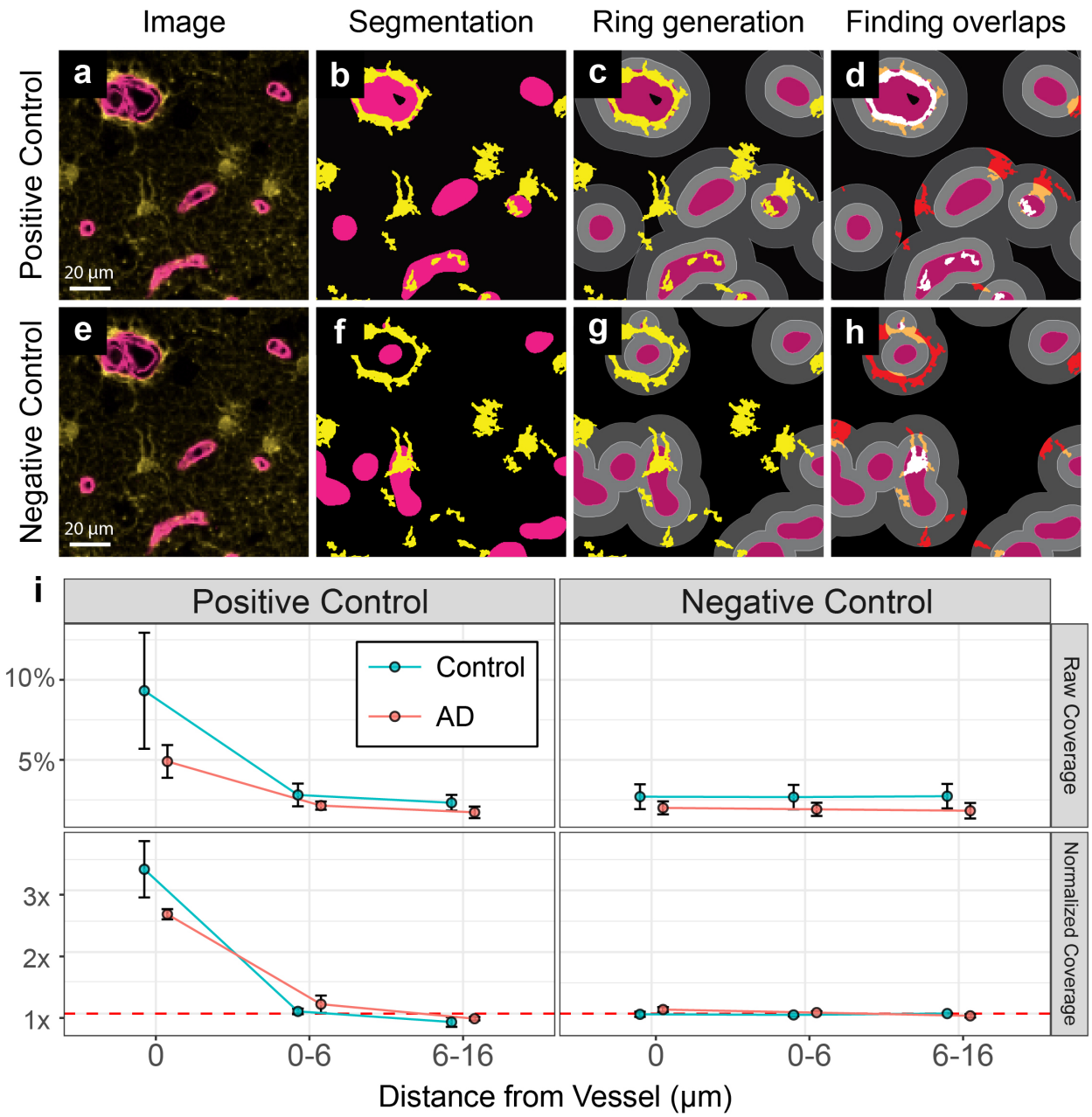
Supplementary Figure S1: Annotations versus detections in QuPath. "Annotation" and "detection" classes of objects in QuPath are not generated with the same default measurements. **(a,b)** MxIF image showing collagen IV (magenta), a marker for blood vessel basement membranes, and DAPI (red), a marker for nuclei. Blood vessels were segmented in QuPath as **(a)** annotation objects (magenta outlines) or **(b)** detection objects (yellow outlines). **(c)** Note the differences in standard metadata included for annotations (magenta box) versus detections (yellow box).



Supplementary Figure S2: Annotating neocortical layers. Markers used to trace neocortical layers manually. The small, white boxes outline areas of the image that were excluded due to tissue loss or staining artifacts. Example of MxIF images in the V1 from a 74-year-old cognitively healthy man, showing each individual marker used to annotate its constituent neocortical layers (white). **(a)** GFAP: layer 1 and white matter. **(b)** HUD: layers 2–6. **(c)** MBP: white matter. **(d)** NPNFP: layers 3, 5 and 6. **(e)** VGAT: layers 2, 3 and 5.



Supplementary Figure S3: Segmenting markers. Example of marker segmentation using QuPath's native thresholding tool. The example shows an MxIF image of DLPFC layer 6 and white matter from an 83-year-old woman diagnosed with AD. **(a)** Channels for collagen IV (magenta) and ALDH1L1 (yellow). **(b)** Same field of view as panel (a) with collagen IV segmented in magenta and ALDH1L1 segmented in yellow.



Supplementary Figure S4: Biological validation of end-to-end workflow. Utilization of randomly placed blood-vessel objects to validate the *AnnoTools* framework and normalization schema. **(a-d)** Framework to measure astrocytic coverage of cerebral blood vessels by astrocytes representing a biological positive control, as outlined in **Fig. 1**. **(e-h)** The same framework applied to randomly placed blood-vessel objects within the same image as a negative control. **(i)** Quantification of the positive and negative controls, shown both as raw and normalized coverage across technical replicates.

Capability	QuPath + *AnnoTools*	QuPath	Fiji	CellProfiler	ilastik	HALO	Squidpy
Whole-slide image visualization	+	+	+	−	</>	+	</>
Image segmentation	+	+	+	+	+	+	</>
Interactive object refinement	+	+	+	</>	</>	+	</>
Custom geometric manipulation	+	</>	+	</>	−	+	</>
Hierarchy-preserving object exchange	+	</>	</>	</>	−	</>	−
Manual multi-reviewer classification	+	</>	</>	−	</>	</>	−
Object-based spatial overlap analysis	+	</>	+	+	</>	+	</>
Advanced spatial statistics	−	</>	</>	</>	</>	+	+
Open-source	+	+	+	+	+	−	+

Supplementary Table S1: Capabilities of *AnnoTools* compared to other commonly used image analysis software. Characters and colors represent features that are available (+), available with additional scripting/programming (</>), and not available (-) for the respective software.

Target	Vendor	Catalog #	RRID	Clone	Working Concentration (µg/ml)
HUD	Santa Cruz	Sc-28299 AF647	AB_10697925	E-1	10
ALDH1L1	Millipore	MABN495	AB_2687399	N103-39	5
GFAP	Sigma	C9205	AB_476829	G-A-5	1
VGAT	Synaptic Systems	131 004	AB_887873	Guinea pig polyclonal	1:400 (working dilution)
NPNFP	Biolegend	SMI-32P	AB_2315332	SMI-32	10
MBP	Abcam	ab7349	AB_305869	12	5
Aβ	Biolegend	803001	AB_2564653	6E10	5
pSer202/ pThr205 Tau	Thermo Fisher	MN1020	AB_223647	AT8	5
Collagen IV	Millipore	MAB3326	AB_11212378	IV-4H12	5

Supplementary Table S2: Antibodies used in MxIF. Full names and targets of antibodies are as follows. For neurons: Hu-antigen D (HUD). For all astrocytes: aldehyde dehydrogenase 1 family member L1 (ALDH1L1). For activated astrocytes: glial fibrillary acidic protein (GFAP). For inhibitory neurons: vesicular GABA transporter (VGAT). For myelinated axons: myelin basic protein (MBP). For nonphosphorylated neurofilament protein-expressing neurons (NPNFP). For amyloid-β/amyloid precursor protein (Aβ). For hyperphosphorylated tau (pSer202/pThr205 Tau). For vasculature: collagen IV.