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Supplementary information

Antigen retrieval and clearing for whole-organ immunofluorescence by FLASH

In the format provided by the authors and unedited

Supplementary information

Supplementary Methods

Microscopy Guide

Considerations on RI-matching. The refractive index (RI) of methyl salicylate (MetSal) is 1.536 and almost no commercial objective lenses are exactly matched for this imaging medium. A mismatch between lens and RI of the medium can exacerbate spherical aberration (rays coming through the edge of the lens get focused closer than rays coming through the middle), chromatic aberration (blue light gets focused closer than red), and axial compression (features along the Z-axis are shortened relative to the XY-plane). The effects of RI mismatch increase with image depth and are more pronounced at high-resolution. For this reason, it is better to use low Numerical Aperture (NA) than high NA detection lenses when imaging in high RI media.

The light-sheet microscope we used for imaging in this study is equipped with a low NA detection lens (2X/0.5 NA) that can be used in media with RI ranging from 1.33 to 1.56 without dramatic optical aberrations. Moreover, a system of motorized tube lens helps to compensate for the dispersion that occurs doing multicolor imaging. Although objectives for confocal microscopy (CM) are commonly made for RI of 1.4, their practical working distance is limited. Moreover, when imaging deep in the tissue with CM and a RI mismatch is present, it is possible to encounter reduction of signal intensity, lower decrease in axial resolution and wrong scaling[1]. In this study high-resolution images obtained with CM suffered from some spherical and chromatic aberration due to the RI mismatch between lens and medium, but were sufficient for extracting information about the sample and the clearing efficiency.

Several companies (Olympus, Zeiss, ASI...) are now developing lenses optimized for imaging in solutions with unconventional RI (BABB, DBE, FocusClear,..), with very long working distance (> 8 mm) and resistance to aggressive solutions. Such lenses could significantly help with imaging of cleared samples in the future.

Considerations on microscope choice. Samples cleared with FLASH can be imaged both with confocal microscopy (CM) and light-sheet microscopy (LSM). Both types of

microscopy have benefits and limitations and it is important to choose the right imaging approach according to the experimental needs. Here we briefly discuss the general differences between CM and LSM.

Speed. In LSM the field of view is typically imaged all at once (all pixels in parallel), whereas in confocal microscopy the field of view is imaged one point at a time (sequentially). This parallelization of signal detection dramatically speeds up LSM acquisition. For example, using confocal microscopy it would take 43 minutes to acquire a 512 x 512 image in which each pixel is exposed for 10 ms, whereas LSM can acquire such an image in approximately 10 ms. Confocal imaging can be sped-up using resonant scanners to scan the sample very rapidly, however the resulting short pixel-illumination time (~10 ns) results in low signal and high noise. LSM further benefits from the use of CMOS cameras which are typically 2 - 5 times more sensitive than the photo-multiplier tubes used for confocal microscopy, allowing the same level of signal to be acquired more rapidly.

Resolution. CM uses single-point scanning illumination and detection, in combination with a pinhole to reject out focus light, which provides better lateral resolution than LSM. Moreover, in LSM the Gaussian light-sheet is not perfectly flat, and the center of the sample (when using double side illumination) does not always receive the same light-dose as the edge, resulting in uneven illumination, non-uniform contrast, and variable resolution across the sample.

For thick samples the axial resolution can in some circumstances be better in LSM than CM. In LSM the axial resolution is determined both by the light-sheet thickness and NA of the detection lens. When a thin light-sheet is coupled with a low NA/low magnification detection lens, LSM can achieve big improvements in axial resolution allowing better optical sectioning.

Sample size. LSM is preferable to CM for imaging large samples (> 1 mm^3) because of its higher speed of acquisition. Moreover, for the LaVision UMII LSM two other factors make it very suitable for big samples: 1) a wide set of sample holders for quickly and easily mounting large and fragile specimens; 2) the long working distance (> 5,7 mm) of the detection lens for imaging thick samples.

Imaging setup and usability. LSM is an evolving microscopy modality, still not widely supported in light microscopy facilities. Some limitations of LSM derive from the fact

that light-sheet microscopes need to be routinely calibrated and the equipment, especially after using organic solvents, needs to be extensively cleaned. However, in term of usability, LSM is not more complicated than CM and in the case of the LaVision UMII the manual optical zoom can dramatically facilitate sample exploration and image acquisition.

Photo-stress. In CM, illumination along the Z-axis results in photo-bleaching above and below the imaging plane. In LSM, illumination 90° to the imaging axis results in photo-bleaching only in the imaging plane. When 3D volumes are acquired by sequential imaging of adjacent planes (Z-stacks), LSM results in much lower photo-bleaching, meaning that images acquired at the beginning and end of the Z-stack are more similar in intensity.

Multicolor imaging. Multicolor imaging with LSM typically relies on bandpass emission filters while in CM other optical tools (using refraction or diffraction) allow more flexible and precise wavelength filtering. Moreover, especially at high resolution chromatic aberration are evident with LSM. For these reasons, when precise colocalization studies or discrimination of fluorophores with overlapping emission spectra are necessary (e.g., GFP and YFP) a CM approach is often preferable.

In summary, LSM is advisable when it is necessary to image thick samples (>1 mm^3), perform medium-low resolution analyses (detect sparse cells or cell populations), quickly explore the specimen or when photo-bleaching can be an issue. CM is preferable for imaging thin samples (<50 μm deep), perform high-resolution (detect single cells or subcellular components) or multicolor colocalization studies. One interesting possibility is to perform correlative LSM-CM microscopy, where a sample is first scanned with LSM and then, if more detailed information is necessary, the same sample (or a part of it) can be re-acquired at high-resolution with CM.

A more detailed comparison of light-sheet microscopy and CM can be found in the works by Jan Huisken[2,3] or in the recent study from Jonkman and colleagues[4].

It is also important to remember that CF and LSM are not the only imaging modalities that can be used for analyzing cleared samples. Other options are available (e.g., two-photon microscopy, confocal spinning disk, structured illumination) but a careful analysis of pros and cons of each modality is necessary.

Guide for staining evaluation

Analyzing a 3D tissue volume by light microscopy bears risks of misidentification of structures due to patchy noise or antibody trapping (e.g. in stromal layers and blood vessels), as well as too high background levels that may obscure true signal. A stained sample that is looked at under the eyepiece, or imaged without optical sectioning, can be prone to misinterpretation. Below are some considerations to take into account when planning an experiment, especially if new labels are to be used.

Label evaluation. Every label comes with background, and it is important to bear in mind that even small levels of background staining in a 2D tissue section can add up in 3D and obscure truly positive structures. Before labels are used on whole organs, they should be tested on a 2D tissue section of the same sample type. We found that antibodies that work well in 2D immunohistochemistry/immunofluorescence on formaldehyde-fixed tissue are likely to give excellent results in FLASH. If an antibody does not work in 2D, it is unlikely to improve with FLASH.

Counterstaining. In addition to the label of interest, each sample should always be co-stained with a well-established marker that detects the same compartment, cell or structure. For example, if the sample is to be stained for a transcription factor, it could be co-stained with a nuclear dye; or if a sample is stained for secretory cells, a pan-epithelial marker could be used in addition. If the label of interest shows background cross-reactivity in conventional 2D staining on tissue sections, this background can mask real signal in 3D. In this case it can help to co-stain the background structures. For example, an antibody against duct cells might potentially be trapped by blood. To the unexperienced, ducts and blood vessels may look similar in 3D as both present arborized structures. In this specific case, co-stain with a vessel marker such as CD31 or α-SMA would help to distinguish true signal from background.

Controls. The controls for 3D imaging are similar to 2D immunofluorescence approaches and depend on the research question. Inclusion of a 2D tissue section helps to evaluate label specificity. Additional single label stainings can be a useful control to inform about antibody crosstalk. Unstained controls are helpful to assess

autofluorescence, which in some cases can be limited to structures or single cells, and may lead to staining misinterpretation if not accounted for.

Staining interpretation. Most current analysis software packages have integrated 2D/3D viewers and it is helpful to evaluate stainings in a back-and-forth between 3D view and optical sections. First, staining specificity should be assessed by comparing optical sections with stained 2D tissue sections, to see if the staining pattern compares. Thereby, the sum tissue fluorescence background informs on the overall sample dimensions; counterstains, such as blood vessels, help as landmarks. This analysis will also detect any tissue damage: Are tissue layers disrupted? Are there gaps in continuous structures like blood vessels or ducts? Is the tissue border intact? In addition to staining specificity, staining distribution should be evaluated by comparing regions close to the sample surface with deeper regions of the same anatomical structures. As explained in the microscopy guide above, especially datasets acquired with confocal microscopy can show a decrease in signal intensity with increasing imaging depth. The consequently decreasing signal-to-noise ratio should be taken into mind in any subsequent analyses.

RI-matching media comparison

Halved pancreata and mammary glands, and the intact smallest liver lobes and lung lobes were treated with FLASH Reagent1 and nuclei stained with Hoechst. For RI-matching in MetSal and BABB, samples were dehydrated in increasing concentrations of MetOH (30%, 50%, 75% and 2 x 100% v/v) and cleared with increasing concentrations of MetSal or BABB in MetOH (25%, 50%, 75%, 2 x 100% v/v). For mounting in DBE, samples were treated as described by Erturk and colleagues[5]. Samples were subjected to 30 min incubations in increasing concentrations of tetrahydrofuran (THF) (50%, 60%, 80% and 3 x 100% v/v), delipidated in dichlormethane (DCM) for 30 min and moved to dibenzyl ether (DBE) overnight. DBE was replaced with fresh DBE the next morning. For mounting in BABB-D4 with delipidation, samples were processed as in Pan *et al*.[6]. Specimens were treated with increasing concentrations of *tert*-Butanol (tB) (30%, 50%, 70%, 80%, 90%, 96% and 100% v/v) at 35°C for 10-16 h per step, delipidated in dichlormethane (DCM) for 70 min at RT and moved to BABB-d4 (4 parts BABB (1:2 benzyl alcohol to benzyl benzoate) to 1 part diphenyl ether) overnight. This solution was exchanged the next

morning. For mounting in CUBIC, samples were incubated in CUBIC reagent 1 for 1 day at 37°C and then incubated in reagent 2 for 1 day. The recipes for both reagents were developed by Susaki *et al*.[7] For mounting in 2,2'-Thiodiethanol (TdE)[8], samples were incubated in increasing concentrations of TdE (30%, 50%, 70% and 97% v/v) for 2 h per step, followed by overnight incubation in 97%.

iDISCO

iDISCO was performed as in Renier *et al.*[9] Briefly, mouse organs were isolated and fixed in 10% NBF for 24 h. Samples were washed in PBS and dehydrated in increasing concentrations of metOH (50%, 80%, 2 x 100% 1 h per step). Samples were bleached overnight in 5% H_2O_2, 20% DMSO in metOH at 4°C overnight. Samples were washed in MetOH 2 x 1 h, 20% DMSO in MetOH 2 x 1 h, and rehydrated in decreasing concentrations of MetOH (80%, 50% 1 h each) and PBS 2 x 1 h. Samples were transferred to 0.2% v/v Triton X-100 in PBS 2 x 1 h. Samples were incubated in 0.2%v/v Triton X-100, 20% v/v DMSO and 0.3 M glycine in PBS at 37°C overnight. Samples were blocked in 0.2% v/v Triton X-100, 10% v/v DMSO and 6% v/v FBS at 37°C for 3 days. Samples were washed in 0.2% Triton X-100 and 10 mg/mL heparin in PBS (PTwH) twice for 1 h, and then incubated in PTwH + 5% DMSO + 3% FBS at with primary antibodies at 37°C for 3 days. Samples were washed in PTwH for 24 h and incubated in PTwH + 3% v/v FBS at 37°C for 3 days. Samples were washed in PTwH for 2 days. Samples were dehydrated in 50% v/v tetrahydrofuran (THF) in water at RT overnight and 80-100-100% v/v THF at RT for 1 hour per step. Samples were incubated in dichloromethane for a few minutes, until they sank to the bottom of the vial. Finally, samples were incubated in dibenzyl ether overnight for clearing.

CUBIC-HistoVIsion

CUBIC-HistoVIsion was performed as in Susaki *et al*.[7] We used the version without enzymatic digestion to avoid degradation of extracellular matrix components and epitope loss. Briefly, mouse organs were isolated and fixed in 10% NBF for 24 h. Samples were incubated in 25% w/v urea, 25% v/v Quadrol, 15% v/v Triton in H_2O rotating gently at 37°C 2 x 3 days. Samples were washed in PBS shaking at 37°C 3 x 2 h and blocked in 10 mM HEPES, 10% v/v Triton X-100, 200 mM NaCl and 0.5% w/v casein (HEPES-TSC) for 1.5 h. Samples were incubated in HEPES-TSC containing primary antibodies at 37°C for 3 days, washed in PBS rocking at RT 3 x 20 min, and

incubated in HEPES-TSC with secondary antibodies for at 37°C for 3 days. Samples were washed in PBS with 10% v/v Triton X-100 for a few minutes and in PBS for 2 hours. Samples were postfixed in 1% NBF at RT for 5h. Samples were incubated in 50% v/v CUBIC-R+(N) (45% w/v antipyrine, 30% w/v nicotinamide and 0.5% v/w N-butyldiethanolamine) in water at RT for 1 day and in 100% CUBIC-R+(N) at RT for 3 days.

SWITCH

SWITCH was performed as in Murray *et al*.[10] Briefly, mouse organs were isolated and fixed in 10% NBF for 24 h. Samples were washed in KHP: 25% v/v 0.1 M HCl and 25% v/v 0.1 M potassium hydrogen phthalate in pH = 3 PBS (50%) for 1 h. Samples were incubated in KHP + 4% v/v glutaraldehyde gently shaking at 4°C for 2 days. Samples were incubated in 4% v/v glutaraldehyde in PBS gently shaking at 4°C for 2 days and 4 hours at 37°C. Samples were then washed in PBS shaking gently at RT for 1 day, and incubated in 4% w/v glycine, 4% w/v acetamide in water at 37°C gently shaking for 1 day. Samples were cleared in 200 mM SDS, 10 mM lithium hydroxide, 40 mM boric acid and 50 mM sodium sulphite, pH = 9, at 65°C for 1 day. Samples were incubated in 10 mM SDS in PBS gently shaking at 37°C for 1 day. Samples were then incubated in primary antibodies in 10 mM SDS in PBS at 37°C for 2 days, washed in PBS rocking at RT 3 x 20 min, and incubated in secondary antibodies in 10 mM SDS in PBS at 37°C for 2 days. Finally, samples were washed in PBS + 0.2% v/v Triton-X100 and mounted in FocusClear™ for imaging.

AbScale

AbScale was performed as in Hama *et al*.[11] Briefly, mouse organs were isolated and fixed in 10% NBF for 24 h. Samples were incubated in 20% w/v D-sorbitol, 5% v/v glycerol 1 mM methyl-b-cyclodextrin, 1 mM g-cyclodextrin, 1% w/v N-acetyl-L-hydroxyproline and 3% v/v DMSO in PBS pH = 7.2 at 37°C for 12 h. Samples were incubated in 10% v/v glycerol, 4 M urea and 0.1% v/v Triton-X-100 in water pH = 7.7 (solution ScaleA2) at 37°C for 36h. Samples were incubated in 8 M urea pH = 8.4 at 37°C for 24 h. Samples were returned to fresh ScaleA2 and incubated at 37°C for 12h. Samples were washed in PBS at RT 3 x 2 h. Samples were washed in PBS 3 x 2 h and incubated in 0.33 M urea, 0.2% Triton X-100 v/v in PBS (AbScale) containing primary antibodies at 37°C for 2 days. Samples were washed in AbScale 2 x 2 h and

incubated in AbScale with secondary antibodies for 48 h. Samples were washed in AbScale rocking at RT for 6 h and rinsed in 2.5% w/v BSA, 0.05% v/v Tween-20 in 0.1 x PBS rocking at RT 2 x 2 h. Samples were refixed in 10% NBF at RT for 1 and washed in PBS rocking at RT for 2 h. Finally, samples were cleared in 40% w/v sorbitol, 10% w/v glycerol, 4 M urea and 20% v/v DMSO pH = 8.1 at 37°C for 1 12 h and at RT until clear.

Untreated MetSal

Mouse organs were collected and fixed in 10% NBF for 24 h. Samples were washed 10 min in PBS and directly stained for 48 h in FLASH blocking buffer with primary antibodies. Samples were incubated for 48 h in secondary antibodies, dehydrated in increasing concentrations of MetOH (30%, 50%, 75% and 2 x 100% v/v) and cleared with increasing concentrations of MetSal in MetOH (25%, 50%, 75%, 2 x 100% v/v).

Quantifications

Heatmap intensity profiles. Stacks across the width of the mammary gland were acquired in the confocal microscope for different clearing techniques. Maximum Intensity Projections (MIPs) of side views of mammary glands were obtained using Imaris Bitplane 8.4.0. Fluorescence intensity heatmaps (LUTs) and calibration bars of MIPs were generated in Fiji.

Imaging depth. Image stacks were acquired on an Andor Dragonfly spinning disk confocal with a 405nm laser and an Andor Zyla 4+ sCMOS camera. Imaging setting were 10um z-step size, 2048x2048 AOI and 2x2 binning. The imaging depth was measured in Imaris as the distance between the first optical section and last optical section where nuclei still could be discerned.

Signal-to-noise ratio (SNR). SNR calculations were performed as in Pan *et al*.[6] in ImageJ. On optical sections immediately below the organ surface a small region was defined, duplicated, a Gaussian blur (sigma=2) applied and the image thresholded (Mean) to separate specific signal from background. The modified image was converted to a mask and values of the mean intensity (for specific signal) and intensity standard deviation (for background pixels) were determined from the image where the region was defined by the mask (by redirecting measurements to the unmodified input area). The background intensity was defined by inverting the mask and the intensity

was measured from the segmented region. The SNR was calculated as SNR=Mean(signal)/Stdev(background).

Nuclear density. The nuclear density was estimated in ImageJ. The scale was set according to dimensions and spatial calibration in the raw images. A small area was selected, thresholded (MaxEntropy), converted to a mask and segmented using the watershed option. The object count per input area was taken as an estimate of nuclear density.

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Supplementary Table 1: comparison of detergents for antigen retrieval					
Detergent	**Abbreviation**	**CAS**	**GBP/gram**	**CMC mM**	**Class**
Sodium dodecyl sulfate	SDS	151-21-3	0.32	7-10	Anionic
Sodium deoxycolate	Na-deoxycholate	302-95-4	1.22	4-8	Anionic
Polysorbate 20	Tween20	9005-64-5	0.24	0.06	Non-ionic
t-Octylphenoxypolyethoxyethanol	Triton-X100	2315-67-5	0.3	0.2	Non-ionic
N-Tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate	Zwittergent 3-10	14933-09-6	9.06	25-40	Zwitterionic
3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate	Chaps	75621-03-3	11.52	6-10	Zwitterionic

Supplementary Table 2: Selected antibodies and lectins validated for FLASH

Primaries (antigens)	Abbreviation	Clonality	Raised in	Catalogue no	Link	Used in	target cell	Organ	Dilution	Recommended protocol	RRID	Also used in
a-smooth muscle actin	SMA	Monoclonal	Mouse	Sigma-Aldrich A5228	ich.com/catalog/product/sigma/a52	Figs. 2b; 3a; 4a, b; 7d, Ext. Dat. Fig. 6a; Ext. Dat. Fig. 7b; Ext. Dat. Fig. 8a	Smooth muscle cells, myofibroblasts, myoepithelium	Several	1:100	Either	Sigma-Aldrich Cat# A5228, RRID:AB_262054	-
a1 Na/K ATPase	ATPase	Monoclonal	Mouse	Abcam ab7671	lpha-1-sodium-potassium-atpase-a	Fig. 3b	Parietal cells	Stomach	1:100	Either	Abcam Cat# ab7671, RRID:AB_306023	-
Acetylated tubulin	Ac-tubulin	Monoclonal	Mouse	Sigma-Aldrich T7451	ich.com/catalog/product/sigma/t745	Ext. Dat. Fig . 2d	Cilia (bronchiolar cells)	Lung	1:100	Either	Sigma-Aldrich Cat# T7451, RRID:AB_609894	-
Amylase	Amy	Polyclonal	Goat	Santa Cruz sc-12821	/www.scbt.com/p/amylase-antibody	Figs. 2b; 3c; Ext. Dat. Fig. 7a	Acinar cells	Pancreas	1:50	Either	Santa Cruz Biotechnology Cat# sc-12821, RRID:AB_633871	-
Aquaporin 1	Aqp1	Polyclonal	Rabbit	Sigma-Aldrich HPA019206	.com/catalog/product/sigma/hpa01	Fig. 4a, b	Endothelial cells	Several	1:100	Either	Sigma-Aldrich Cat# HPA019206, RRID:AB_1844965	-
Aurora B	AurB	Monoclonal	Mouse	BD 611082	.bdbiosciences.com/ptProduct.jsp?	Fig. 6c; 8b	Cytoskeleton	Several	1:100	FLASH2	BD Biosciences Cat# 611082, RRID:AB_2227708	Tedeschi et al., Nat Commun, 2020
Cadherin 1	Cdh1	Monoclonal	Rat	Thermo Fisher 13-1900	ody/product/E-cadherin-Antibody-c	Fig. 3b, 4d	Epithelial cells	Several	1:100	Either	Thermo Fisher Scientific Cat# 13-1900, RRID:AB_2533005	-
CD16	CD16	Monoclonal	Mouse	Thermo Fisher MA1-7633	body/product/CD16-Antibody-clone	Fig. 5f	Macrophages	Several	1:100	Either	Thermo Fisher Scientific Cat# MA1-7633, RRID:AB_2103889	-
CD3	CD3	Polyclonal	Rabbit	Abcam ab5690	ww.abcam.com/cd3-antibody-ab56	Fig. 5f	T cells	Several	1:100	Either	Abcam Cat# ab5690, RRID:AB_305055	-
CD31	CD31	Polyclonal	Rabbit	Abcam ab28364	om/cd31-antibody-ab28364.html#t	Fig. 5d, Ext. Dat. Fig. 8b	Endothelial cells	Several	1:100	Either	Abcam Cat# ab28364, RRID:AB_726362	-
CD44	CD44	Monoclonal	Rat	Merck MAB2137	millipore.com/GB/en/product/msds/	Fig. 4a	Bile duct cells	Liver	1:100	Either	Millipore Cat# MAB2137, RRID:AB_2076454	-
CD45R	B220	Monoclonal	Rat	Biolegend 103202	/search-results/purified-anti-mouse	Fig. 5f	Immune cells	Several	1:100	Either	BioLegend Cat# 103202, RRID:AB_312987	-
Clara Cell secretory protein	CC10	Polyclonal	Goat	Santa Cruz sc-9772	s://www.scbt.com/p/cc10-antibody-	Fig. 3a	Club cells	Lung	1:100	Either	Santa Cruz Biotechnology Cat# sc-9772, RRID:AB_2238819	-
Cleaved caspase 3	CC3	Polyclonal	Rabbit	R&D AF835	om/products/human-mouse-active-	Fig. 8a	Apoptotic cells	Several	1:100	Either	R and D Systems Cat# AF835, RRID:AB_2243952	Tedeschi et al., Nat Commun, 2020
Collagen IV	ColIV	Polyclonal	Goat	Sigma-Aldrich AB769	ich.com/catalog/product/mm/ab769	Fig. 3c, d; 5b, c, d; 7a; Ext. Dat. Fig. 8a	ECM	Several	1:50	Either	Abcam Cat# ab769, RRID:AB_306025	-
Conecting peptide	C-pep	Polyclonal	Rabbit	CST 4593	.com/products/primary-antibodies/c	Ext. Dat. Fig. 7a	Islets of Langerhans	Pancreas	1:100	Either	Cell Signaling Technology Cat# 4593, RRID:AB_10691857	-
Cytochrome P450	Cyt P450	Monoclonal	Mouse	Abcam ab22717	p450-1a2-antibody-d15-16vii-f10f12	Ext. Dat. Fig. 2b	Microsomes (hepatocytes)	Liver	1:100	FLASH2	Abcam Cat# ab22717, RRID:AB_447282	-
Cytokeratin 19	Krt19	Monoclonal	Rat	DSHB TROMA-III	s://dshb.biology.uiowa.edu/TROMA	Fig. 4a, b; 7a; Ext. Dat. Fig. 7a, b	Ductal cells	Pancreas	1:100	Either	DSHB Cat# TROMA-III, RRID:AB_2133570	Messal et al., Nature, 2019
Cytokeratin 5	Krt5	Monoclonal	Mouse	Biotechne NBP2-22194	o.com/products/cytokeratin-5-antib	Fig. 3d	Basal cells	Mammary gland	1:100	Either	Novus, Cat# NBP2-22194, RRID:AB_2857967	-
Cytokeratin 8	Krt8	Monoclonal	Rat	DSHB TROMA-I	ps://dshb.biology.uiowa.edu/TROMA	Fig. 3d; 5, 7 c-e; Ext. Dat. Fig. 8a, b; Ext. Dat. Fig. 9a	Luminal cells	Mammary gland	1:100	Either	DSHB Cat# TROMA-I, RRID:AB_531826	-
Forkhead box protein P1	FoxP1	Polyclonal	Rabbit	CST 2005	l.com/products/primary-antibodies	Ext. Dat. Fig. 8c	Nucleus (TF)	Mammary gland	1:100	FLASH2	Cell Signaling Technology Cat# 2005, RRID:AB_2106979	-
Gastric Intrinsic Factor	GIF	Polyclonal	Rabbit	Sigma-Aldrich HPA040774	.com/catalog/product/sigma/hpa040	Fig. 3b	Murine chief cells	Stomach	1:100	Either	Sigma-Aldrich Cat# HPA040774, RRID:AB_10795626	-
Glial Fibrillary Acidic Protein	GFAP	Polyclonal	Rabbit	Abcam ab7260	ww.abcam.com/gfap-antibody-ab7	Ext. Dat. Fig . 6a	Astrocytes	Brain	1:100	Either	Abcam Cat# ab7260, RRID:AB_305808	-
Glutamine synthetase	GS	Polyclonal	Rabbit	Abcam ab73593	m.com/glutamine-synthetase-antib	Fig. 4a	Pericentral hepatocytes	Liver	1:100	Either	Abcam Cat# ab73593, RRID:AB_2247588	-
Green Fluorescent Protein	GFP	Polyclonal	Goat	Abcam ab6673	ww.abcam.com/gfp-antibody-ab66	Fig. 5g, Ext. Dat. Fig. 4 b, e	Reporter	-	1:100	Either	Abcam Cat# ab6673, RRID:AB_305643	Messal et al., Nature, 2019
Keratin 14	Krt14	Monoclonal	Mouse	Abcam ab9220	m.com/cytokeratin-14-antibody-rck	Fig. 4b	Basal cells	Mammary gland	1:100	Either	Abcam Cat# ab9220, RRID:AB_307087	-
Mist1	Mist1	Monoclonal	Mouse	Santa Cruz sc-80984	s://www.scbt.com/p/mist1-antibody	Fig. 3c	Nucleus (TF)	Several	1:100	FLASH2	Santa Cruz Biotechnology Cat# sc-80984, RRID:AB_2065216	-
Mucin-1	Muc1	Polyclonal	Rabbit	Abcam ab15481	ww.abcam.com/muc1-antibody-ab1	Fig. 3b	Glandular cells	Several	1:100	Either	Abcam Cat# ab15481, RRID:AB_301891	-
Mucin-5AC	Muc5AC	Polyclonal	Goat	Santa Cruz sc-16903	/www.scbt.com/p/mucin-5ac-antibod	Fig. 3b; 4d	Glandular cells	Several	1:100	Either	Santa Cruz Biotechnology Cat# sc-16903, RRID:AB_649616	-
Neuroendocrine convertase 1	PCSK1	Polyclonal	Rabbit	Millipore SAB1100416	.com/catalog/product/sigma/sab110	Fig. 2b	Islets of Langerhans	Pancreas	1:100	Either	Sigma-Aldrich Cat# SAB1100416, RRID:AB_10606261	-
Podoplanin	Pdpn	Polyclonal	Goat	R&D AF3244	ems.com/products/mouse-podopla	Fig. 7d, e	Alveolar cells	Lung	1:50	Either	R and D Systems Cat# AF3244, RRID:AB_2268062	-
Proliferating cell nuclear antigen	PCNA	Polyclonal	Rabbit	Santa Cruz sc-7907	s://www.scbt.com/p/pcna-antibody-f	Fig. 6b, c	Proliferative cells	Several	1:100	Either	Santa Cruz Biotechnology Cat# sc-7907, RRID:AB_2160375	-
Proliferation marker Ki67	Ki67	Monoclonal	Rabbit	Abcam ab16667	y.abcam.com/ki67-antibody-sp6-ab	Fig. 6a	Cells in G1 to G2	Several	1:100	Either	Abcam Cat# ab16667, RRID:AB_302459	-
Prospero homeobox protein 1	Prox1	Polyclonal	Rabbit	Abcam ab101851	w.abcam.com/prox1-antibody-ab10	Fig. 4a	Lymphatic endothelial cells	Several	1:100	FLASH2	Abcam Cat# ab101851, RRID:AB_10712211	-
Red Fluorescent Protein	RFP	Polyclonal	Rabbit	Rockland 600-401-379	bodies-to-GFP-and-Antibodies-to-F	Fig. 3d	Reporter	-	1:100	Either	Rockland Cat# 600-401-379, RRID:AB_2209751	Messal et al., Nature, 2019
S100	S100	Polyclonal	Rabbit	Dako Z0311	istochemistry/antibodies-controls/s	Fig. 4a, b	Nerves	Several	1:100	Either	Agilent Cat# Z0311, RRID:AB_10013383	-
Surfactant Protein C	SFTPC	Polyclonal	Rabbit	Sigma-Aldrich HPA010928	.com/catalog/product/sigma/hpa010	Fig. 3a; 7d	Alveolar type II cells	Lung	1:100	Either	Sigma-Aldrich Cat# HPA010928, RRID:AB_1857425	-
Tubulin	Tub	Monoclonal	Rat	Abcam ab6161	/tubulin-antibody-yol134-microtubu	Fig. 8b; Ext. Dat. Fig. 2a, d	Cytoskeleton	Several	1:50	FLASH2	Abcam Cat# ab6161, RRID:AB_305329	Tedeschi et al., Nat Commun, 2020
Tyrosine hydroxylase	TH	Polyclonal	Rabbit	Merck AB152	GB/en/product/Anti-Tyrosine-Hydro	Fig. 2c; 5b, c; Ext. Dat. Fig.. 6b; Ext. Dat. Fig. 7b	Neurons	Several	1:100	Either	Millipore Cat# AB152, RRID:AB_390204	-
Vimentin	Vim	Polyclonal	Chicken	Sigma-Aldrich AB5733	ich.com/catalog/product/mm/ab573	Fig. 4b	Mesenchymal cells	Several	1:100	Either	Millipore Cat# AB5733, RRID:AB_11212377	-
Wilms Tumour 1	WT1	Polyclonal	Rabbit	Santa Cruz sc-192	s://www.scbt.com/p/wt1-antibody-c	Fig. 4c	Glomerular cells	Kidney	1:100	FLASH2	Santa Cruz Biotechnology Cat# sc-192, RRID:AB_632611	-
Lectins	**Abbreviation**	**Clonality**	**Raised in**	**Catalogue no**	**Link**	**Used in**	**target cell**	**Organ**	**Dilution**	**Recommended protocol**	**RRID**	**Also used in**
DBA-FITC (lectin)	-	-	-	Vector labs FL-1031	n/fluorescein-labeled-dolichos-biflor	Fig. 4a, c; Ext. Dat. Fig. 1	-	-	-	Either	Vector Laboratories Cat# FL-1031, RRID:AB_2336394	Messal et al., Nature, 2019
DBA-Rhodamine (lectin)	-	-	-	Vector labs RL-1032	n/rhodamine-labeled-dolichos-biflor	Fig. 4a, c; 7b	-	-	-	Either	Vector Laboratories Cat# RL-1032, RRID:AB_2336396	-
PNA-FITC (lectin)	-	-	-	Vector labs FL-1071	s.com/fluorescein-labeled-peanut-ag	Fig. 4c	-	-	-	Either	Vector Laboratories Cat# FL-1071, RRID:AB_2315097	-
Conjugated antibodies	**Abbreviation**	**Fluorophore**	**Raised in**	**Catalog no**	**Link**	**Used in**	**target cell**	**Organ**	**Dilution**	**Recommended protocol**	**RRID**	**Also used in**
Anti-Chicken IgY	-	FITC	Donkey	Thermo Fisher SA1-72000	roduct/Donkey-anti-Chicken-IgY-H-L-	Fig. 4b	-	-	1:250	Either	Thermo Fisher Scientific Cat# SA1-72000, RRID:AB_923386	-
Anti-goat IgG	-	Alexa Fluor 546	Donkey	Thermo Fisher A-11056	t/Donkey-anti-Goat-IgG-H-L-Cross-Ad	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-11056, RRID:AB_2534103	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-goat IgG	-	Alexa Fluor 647	Donkey	Thermo Fisher A-21447	t/Donkey-anti-Goat-IgG-H-L-Cross-Ad	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-21447, RRID:AB_2535864	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-mouse IgG	-	Alexa Fluor 488	Donkey	Thermo Fisher A-11055	/product/Donkey-anti-Mouse-IgG-H-	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-11055, RRID:AB_2534102	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-mouse IgG	-	Alexa Fluor 546	Donkey	Thermo Fisher A-10036	onkey-anti-Mouse-IgG-H-L-Highly-Cro	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A10036, RRID:AB_2534012	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-mouse IgG	-	Alexa Fluor 594	Donkey	Thermo Fisher A-21203	onkey-anti-Mouse-IgG-H-L-Highly-Cro	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-21203, RRID:AB_2535789	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-mouse IgG	-	Alexa Fluor 700	Goat	Thermo Fisher A-21036	ct/Goat-anti-Mouse-IgG-H-L-Cross-Ad	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-21036, RRID:AB_2535707	-
Anti-rabbit IgG	-	Alexa Fluor 546	Donkey	Thermo Fisher A-10040	onkey-anti-Rabbit-IgG-H-L-Highly-Cro	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A10040, RRID:AB_2534016	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-rabbit IgG	-	Alexa Fluor 594	Donkey	Thermo Fisher A10040	onkey-anti-Rabbit-IgG-H-L-Highly-Cro	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A10040, RRID:AB_2534016	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-rabbit IgG	-	Alexa Fluor 647	Donkey	Thermo Fisher A-31573	onkey-anti-Rabbit-IgG-H-L-Highly-Cro	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-31573, RRID:AB_2536183	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-rat IgG	-	Alexa Fluor 488	Donkey	Thermo Fisher A-21208	Donkey-anti-Rat-IgG-H-L-Highly-Cross	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-21208, RRID:AB_2535794	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-rat IgG	-	Alexa Fluor 594	Donkey	Thermo Fisher A-21209	Donkey-anti-Rat-IgG-H-L-Highly-Cross	Several	-	-	1:100 - 1:1000	Either	Thermo Fisher Scientific Cat# A-21209, RRID:AB_2535795	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
Anti-rat IgG	-	Alexa Fluor 647	Donkey	Abcam 150155	/donkey-rat-igg-hl-alexa-fluor-647-pr	Several	-	-	1:100 - 1:1000	Either	Abcam Cat# ab150155, RRID:AB_2813835	Messal et al., Nature, 2019; Tedeschi et al., Nat Commun, 2020
GFP sdAb - FluoTag-Q	GFP nanobody	ATTO 488	Alpaca	SYSY N0301-At488-S	ps://sysy.com/product/N0301-At488	Extended Data Fig. 4 c, f	Reporter	-	1:100	Either	Synaptic Systems Cat# N0301-At488-S, RRID:AB_2744617	-

Supplementary Table 3: procedure recommendation according to sample type

Sample	Species	Depigmentation	Antigen retrieval	Volume of antibody solution (uL)	Antibody incubation	Microscope	Figure reference
Organoids	Mouse	No	FLASH2	200	Overnight (1ary) 1h (2ary	Confocal	Fig. 6
<500 um tissue slices	Mouse	Yes if pigmented	Depends on density	500	2 days	Confocal	Fig. 4a; 5b, c; 8b; Ext. Dat. Fig. 2; 6
<E14.5 whole embryos	Mouse	Yes	FLASH2	500	2 days	Light-sheet	Fig.8
Whole lung lobes	Mouse	No*	FLASH1	500	2 days	Depends on desired resolution	Fig. 4a; 7d, e
Whole pancreas	Mouse	No*	FLASH1	1000	2 days	Depends on desired resolution	Fig. 2b; Ext. Dat. Fig. 7
Whole mammary gland	Mouse	No*	FLASH1	1000	2 days	Depends on desired resolution	Fig. 3d; Ext. Dat. Fig. 8; 9
Whole lacrimal gland	Mouse	No*	FLASH1	500	2 days	Depends on desired resolution	Fig. 4b
Whole fly	Drosophila	Yes, specific protocol	FLASH1	500	2 days	Confocal	Fig. 5g
Intestine fragment	Mouse	No*	FLASH1	500-2000 depending on size	2 days	depends on desired resolution	-
<500 um tissue slices	Human	Yes	Depends on density	500	3 days	Confocal	Fig. 4d
Whole kidney	Mouse	Yes	FLASH2	1000	3 days	Light-sheet	Fig. 4c
Whole spleen	Mouse	Yes	FLASH2	1000	3 days	Light-sheet	Fig. 5e, f
Whole heart	Mouse	Yes	FLASH2	2000	3 days	Light-sheet	Fig. 5 b-d
1/2 stomach	Mouse	No*	FLASH1	2000	3 days	Light-sheet	Fig. 3b
Whole liver lobe	Mouse	Yes	FLASH2	2000-4000 depending on size	4 days	Light-sheet	Fig. 4a
Whole tumour/large piece	Mouse	No*	Depends on density	1000-4000 depending on size	4 days	Light-sheet	Fig. 7 a-e
		*Recommended if not perfused					

Supplementary Table 4: comparison of FLASH with other 3D imaging techniques								
Technique	**Mammary gland cleared?**	**Pancreas cleared?**	**Brain cleared?**	**Time**	**Dehydration**	**Endogenous fluorescence**	**Original reference**	**Comments**
FLASH	Yes	Yes	Yes	7 days	Yes	Incompatible with MetSal	Messal et al., Nature, 2019	Mild sample shrinkage
AbScale	No	No	Yes	9 days	No	Compatible	Hama et al, Nat Neurosci, 2015	Mild sample enlargement
SWITCH	No	No	Yes	11 days	No	Untested	Murray et al., Cell, 2015	Antigen labelling unsuccessful in the timescales used in this paper
CUBIC HistoVIsion	No	Yes*	Yes	17 days	No	Compatible	Susaki et al., Nat Commun, 2020	Mild sample enlargement
iDISCO	Yes	Yes*	Yes	17 days	Yes	Incompatible	Renier et al., Cell, 2014	Toxic clearing reagents. Mild sample shrinkage
		*immunolabelling unsuccessful						