ADAPT-3D: Accelerated Deep Adaptable Processing of Tissue for 3-Dimensional Fluorescence Tissue Imaging for Research and Clinical Settings

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Running title: fast aqueous tissue clearing

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Summary

Light sheet microscopy and preparative clearing methods that improve light penetration in 3D tissues have revolutionized imaging in biomedical research. Here we present ADAPT-3D, a streamlined 3-step approach to turn tissues optically transparent while preserving tissue architecture with the versatility to handle diverse tissue sizes and types across species. Unlike extensive lipid removal utilized by existing protocols, ADAPT-3D only partially removes lipids to preserve cell membranes, yet the non-toxic aqueous refractive indexing solution still rapidly turns tissues transparent while preserving the fluorescence of endogenous and antibody conjugated fluorophores. ADAPT-3D prepares whole mouse brains for light sheet microscopy in a 4-hour refractive indexing step after less than 4 days of preprocessing without changing their size. By maintaining tissue size, ADAPT-3D clears 1-mm thick brain slices in under 24 hours without causing damage and facilitates a 3D section-like view of the meandering choroid plexus. We applied ADAPT-3D to overcome challenges of whole mouse skull clearing and visualized the undisturbed brain borders including specialized skull channels after just 8 days of tissue preparation. ADAPT-3D also had utility in clearing and immunolabeling human intestinal tissues in about 5 days. Overall, ADAPT-3D provides a high-speed, non-shrinking, and fluorescence-preserving workflow for 3D imaging that bridges section-based and whole-organ studies, offering new opportunities for biological discovery.

Introduction

Over the last decade, interest in 3D fluorescence tissue imaging has flourished, accompanied by the advance of several protocols to refine approaches. While some new imaging modalities like light-sheet microscopy[1] have contributed to the advances, there have been many refinements in tissue processing to optimize image acquisition using established modalities like tile-scanning confocal microscopy. In 2013, a novel method called CLARITY that aimed to improve light penetration and reduce light diffraction was introduced in which electrical current, urea, and detergents were applied at length to physically remove lipids from organs like mouse brain.[2, 3] To preserve tissue integrity under these conditions, perfusion fixation and penetration of the tissue with a hydrogel was recommended. While this method worked well with endogenous fluorescent reporters expressed in mouse, antibody penetration through the gel was limited but has improved in further modifications.[4-6] The introduction of CLARITY coincided with a revived focus on 3D reconstruction of tissue structures, as 3D imaging of long, thin structures like vessels and nerves yields much more informative biology than thin sections.

These reinvigorated efforts in 3D imaging included use of solutions with a refractive index that matches the surrounding tissue to reduce the diffraction of light through the sample and thereby improve light penetration and collection. This practice, referred to as refractive index matching (RIM), was introduced over a century ago when organic mixtures of plant-derived essential oils, methyl salicylate and benzyl benzoate, were applied to fully dehydrated tissue samples that had been decalcified.[7] Oils like methyl salicylate, for example, have the capacity to match refractive index sufficiently well that light will pass through >1 millimeter slices of opaque adipose tissue efficiently enough to generate a gross appearance that the fat is as clear to the eye as "glass" [8], though little to no extraction of fats or molecules occurs. Novel organic solvent-based techniques called iDISCO (and variants like iDISCO+)[9-11] and methods based on use of the essential oil ethyl cinnamate[12, 13] have emerged as powerful ways to achieve RIM from mouse organs to pig brains. Because these methods use organic solvents, they shrink and may distort tissue or denature fluorescent molecules, thereby hindering use in some applications.

Other historical studies demonstrated promising results in RIM were obtained with glycerol.[14] Over time, the impact of sugars in altering the properties of light scattering became increasingly recognized,[15-17] supporting an interest in aqueous mixtures for RIM. In contrast to organic solvents, aqueous solutions hold potential to avoid shrinkage of tissues during dehydration and may be less denaturing to fluorescent molecules. Robust RIM can be achieved in an aqueous setting using solutions containing x-ray contrast reagents like iohexol (brand name Nycodenz) or iodixanol, which have a high refractive index (RI, >1.4). These compounds have a proven safety profile given their use as X-ray or computed tomography imaging contrast reagents such as those found in Ce3D.[18, 19] Urea is also useful (RI > 1.3), given its hyperhydrating property.[16, 20-22] Combinations of X-ray contrast reagents like iohexol and high concentrations of urea allow for faster-acting, aqueous RIM protocols, like CUBIC with the RIM step taking 7 or more days,[20] Fast 3D Clear with an RIM step of 3 days,[23] or EZ Clear that achieves RIM of most tissues within 24 h.[24] Since RIM is only one of a few steps in tissue preparation for imaging, the tissue harvest to imaging time frame is longer for each of these methods than the length of this single step.

Combining RIM protocols with methods that extract lipids and other light-interfering molecules from tissues further improves depth and clarity of 3D imaging. Delipidation methods exist, each with trade-offs in speed, complexity, and tissue preservation. Organic solvent-based

methods such as iDISCO+ or SHANEL, for example, employ small-micelle detergents like CHAPS or salt-free amines for effective lipid extraction combined with dehydration through graded alcohols with overnight incubation in dichloromethane, a potent delipidating agent, before refractive index matching in organic media [9, 18, 23, 25]. These protocols are fast—often clearing tissues overnight—but involve multiple handling steps and can lead to tissue shrinkage. Aqueous approaches utilize cationic detergents, such as N-alkylimidazole and the cross-linking agent Quadrol (N,N,N′,N′-Tetrakis(2-hydroxypropyl)ethylenediamine) that is found in CUBIC, which efficiently remove pigments like heme from tissue to further facilitate light penetration and reduce background fluorescence.[18, 20] The use of cationic detergents (e.g., N-alkylimidazole) or tertiary amines (i.e., N-butyldiethanolamine) combined with detergents like TritonX-100 efficiently remove lipids like heme and improve light penetration, but take several days. While this extended clearing improves labeling accessibility, aggressive lipid removal can distort morphology. In some contexts, tissue swelling caused by CUBIC may help enhance cellular resolution,[18, 26] but this risks loss of structural fidelity.

We took inspiration from these many advances in aqueous-based clearing methods for our 3D tissue imaging applications. In so doing, we also encountered obstacles, like the tendency of urea to crystallize when it was present at high concentrations in clearing solution. To that end, we undertook efforts to further optimize aqueous approaches to achieve rapid, aqueous 3D imaging. We refer to our new solutions and protocols as ADAPT-3D, Accelerated Deep Adaptable Processing of Tissue for 3-Dimensional imaging. Rather than taking days to achieve RIM, our protocol achieves uniform RIM in minutes to hours for most tissues. We couple this RIM step with optimized fixation, decolorization, and delipidation. ADAPT-3D minimizes tissue distortion and the possibility of complications like formation of urea crystals. A version of our method includes efficient decalcification for imaging bone attached to soft tissues.

RESULTS AND DISCUSSION

Development of ADAPT-3D

Recent literature to develop aqueous, fast-acting clearing, or RIM (refractive index matching), solutions while minimizing toxic chemicals focused on combinations of x-ray contrast solution with urea [23, 24]. Fast action was reported to be 2-3 days for the RIM step alone, not including additional time for staining [23, 24]. A solution of 7M urea in 80% iohexol in a method referred to as EZ Clear was especially appealing [24]. However, the preparation of the EZ Clear mixture for RIM is lengthy and requires heating. Furthermore, the solution is subject to crystallization, including after being applied to tissue, due to urea being near its saturation point especially as imaging time lengthens. We aimed to find an improved solution while working a similar theme of chemicals. Combinations of components were tested to achieve optical clarity in tissues within hours, preservation of signal-to-noise, retention of endogenous fluorescent tracers, low toxicity, minimal browning from Maillard reactions, a refractive index as high as possible, maintenance of tissue morphology, and ease of preparation and use. We determined that iodixanol could be used as a base solution, allowing us to start at a refractive index of 1.43 and to proceed without the need for heating when other chemicals were added. To counteract tissue swelling that occurred with iodixanol used alone, we paid close attention to the inclusion of sugars that would partially dehydrate tissue and thereby offset the expansion effect of iodixanol. Although we aimed to reduce the high, crystallization-prone urea concentration associated with EZ Clear, we

observed that inclusion of some urea accelerated the speed at which the solution permeated the tissue to achieve RIM. Ultimately, a particular combination of sucrose (30% wt/vol), urea (25% wt/vol), iohexol (~26% wt/vol, alternatively known as Histodenz) dissolved into commercially prepared iodixanol (alternatively known as OptiPrep) satisfied all desired characteristics outlined above and resulted in refractive index of 1.50-1.51. We named this formulated RIM solution ADAPT:RI (Table 1). For a complete preparatory workflow for passive immersion of samples, we formulated four additional solutions: (i) a fixative that preserves the fluorescence of endogenous fluorophores (ADAPT:Fix), (ii) a decolorization buffer to remove pigments (ADAPT:DC), (iii) a gentle delipidation buffer for partial removal of lipids (ADAPT:PDL), (iv) and a decalcification buffer for bones (ADAPT:Decal, Table 1).

As an initial step to prepare samples for processing across diverse applications, we optimized fixation conditions to retain intensity of sensitive endogenous fluorescent reporters while limiting masking of antigens that would be detected by immunolabeling. Based on prior studies using recombinant fluorescent reporter proteins (FP), we investigated how altering pH, temperature, and adding sugars might augment the intensity of the FPs [27-29]. Systematic surveys of different fixation conditions using 4% w/v paraformaldehyde (PFA) as the base fixative were performed by measuring the fluorescent intensity of lymphoid follicles containing CD11c-eYFP^{+} cells within Peyer's Patches of mice (Fig. 1A). The lowest average CD11c-eYFP signal intensity was observed after fixing tissue with the most widely used formulation of 4% PFA at neutral pH 7.6 (Fig. 1A). As is well known, the addition of the known protein stabilizing agent sucrose to a concentration of at least 10% (w/v) further improved retention of fluorescence intensity (Fig. 1A). Adjustment to pH 9.0 alone also provided a significant retention of signal intensity. Addition of at least 10% (w/v) sucrose to 4% (w/v) PFA at pH 9.0 was as efficient for preserving fluorescence intensity of eYFP as 30% sucrose at pH 7.6 (Fig. 1A). For some tissues, however, fixation with 30% sucrose was sufficiently dehydrating as to cause tissue compression. We thus proceeded with use of 4% PFA, pH 9.0 with 10% sucrose, referring to it as ADAPT:FIX (Table 1).

To test the utility of the ADAPT:RI solution for RIM, we evaluated whether use of the solution alone could render full-thickness samples of human, piglet, and mouse colon transparent. ADAPT:RI alone rendered mouse colon (600 μm) partially transparent while the presence of endogenous pigments prevented full transparency (Fig. 1B, right). The light scattering effects of pigments and lipids were further magnified in thicker human and piglet colon tissue (1.5–2.0 mm) where only slight translucency was achieved (Fig. 1C, Supplemental Fig. 1A). After removal of pigments using the previously published SHANEL decolorization solution containing CHAPS and N-methyldiethanolamine[25], ADAPT:RI rendered mouse tissue visibly transparent to the naked eye within 10 minutes at room temperature (Fig. 1B, left).

To generate a faster-acting decolorant, we adapted the previously the SHANEL decoloring solution[25] by adding limited amounts of N-butyldiethanolamine to increase tissue permeability while preserving morphology and 1,2 hexanediol, an inert emulsifying and humidifying agent (Table 1). To develop the solutions of ADAPT-3D, we consulted *in silico* resources including those from previous chemical screening used to generate the CUBIC-L protocol [18], Reaxys, Open Reaction Database, and WolframAlpha for potential unwanted reactivity between chemicals and found none. We noted that the preparation of ADAPT:DC did not generate a notable exothermic or endothermic reaction. Reagents like hexanediol are used in skin and hair care products and thus we deemed them to have a favorable safety profile. In a comparison of decolorization alone (without RIM solution), ADAPT:DC achieved greater

transparency of piglet colon relative to the previously formulated SHANEL decolorization[25] over the same 80-minute timeframe (Supplemental Fig. 1B).

To promote partial removal of light-interfering lipids, we aimed to develop a protocol that, while removing tissue lipids, would not lead to significant shrinkage that is caused by some solutions, such as graded exposure to tetrahydrofuran[23] that has been long recognized for its delipidation properties.[9, 30, 31] Although shrinkage can be minimized with a multistep gradient of tetrahydrofuran and reversed by rehydration [23], any initial tissue shrinkage can cause irreversible structural tears in tissue. We predicted that combining tetrahydrofuran with a known delipidating agent 1,2-hexanediol [32] that also has hydrating properties would create an effective single step delipidation while preserving tissue size. Following an initial 80-minute decolorization step, treatment of mouse and pig colons with a balanced mixture of tetrahydrofuran and 1,2-hexanediol (Table 1) for 2 hours enabled effective ADAPT:RI of the mouse colon within 30 minutes (Fig. 1D, top row) while the thicker piglet colon took 1 hour to achieve visible transparency (Fig. 1D, bottom row).

Although lipids are present in all tissues, including the intestine, we next evaluated the efficacy of ADAPT:PDL for preparing lipid-rich white matter regions of the mouse brain for RIM. In a 1-mm thick brain slice from a $LysM^{Cre}$;$tdTomato^{fl/fl}$ mouse (Fig. 1E), ADAPT:DC alone without ADAPT:PDL was insufficient to achieve complete optical transparency after RIM particularly in the lipid-rich white matter (Fig. 1F). However, a short 3-hour incubation in ADAPT:PDL enabled complete visible transparency by ADAPT:RI (Fig. 1G). Decolorization alone also improved transparency after RI matching possibly due to the moderate delipidating effects of CHAPS, N-butyldiethanolamine, and 1,2-hexanediol in ADAPT:DC in addition to the removal of pigments (Supplemental Fig. 1C). To assess how visual differences in tissue transparency affected 3D fluorescent imaging, we acquired full thickness z-stacks from select areas in the $LysM^{Cre}$;$tdTomato^{fl/fl}$ 1-mm brain slice with nuclear labeling (Fig. 1H). In the brain stem, which was visibly opaque without delipidation, fluorescent signal from nuclei labeled with anti-Histone-Atto488 was only detected to a depth of 150 μm (Fig. 1I, left). A 3-hour step with ADAPT:PDL enabled clear nuclear detection throughout the full thickness of the 1-mm slice (Fig. 1I, right). While the cortex appeared more transparent by eye, fluorescence imaging revealed reduced nuclear signal detection at depth, with near-complete loss beneath the lipid-rich corpus callosum (Fig. 1J, left). Delipidation restored uniform nuclear detection across the entire cortex, including the corpus callosum (Fig. 1J, right).

Having established ADAPT:PDL as a key component of the broader ADAPT-3D workflow in brain slices, we next evaluated its clearing speed and ability to preserve tissue size in whole mouse brains relative to the widely used organic-based iDISCO method. The iDISCO+ protocol, involving five steps with methanol dehydration and incubation with dichloromethane, cleared fixed brains in 4 days but caused ~40% shrinkage (Fig. 1K), consistent with previous reports of tissue shrinkage[33] and detachment from the dural membrane in whole skull clearing[34]. In contrast, two days of the single-step ADAPT-3D delipidation following 1 overnight incubation in decolorization facilitated successful ADAPT-3D RIM of the fixed brain in 4 hours without causing shrinkage (Fig. 1L). By combining the delipidating power of tetrahydrofuran with the hydrating and delipidating properties of 1,2-hexanediol, the ADAPT-3D buffer matched the speed of iDISCO, while eliminating shrinkage and reducing the number of handling steps.

We applied our newly formulated ADAPT-3D four step protocol of fixation, decolorization, delipidation, and RIM (Supplemental Fig. 1D) to make a variety of tissues transparent including, but not limited to, heme-rich spleen (Supplemental Fig. 1E), lung

(middle), and a liver from a mouse with endogenously fluorescent hepatocytes and lymphatic vessels (i.e., Prox1^{CreER};TdTomato$^{fl/fl}$ reporter mice) such that the transparent tissue retained red intensity from the tdTomato reporter (Supplemental Fig. 1E, right panel). In summary, these solutions collectively supported rapid tissue processing for 3D imaging while avoiding significant changes in tissue morphology at all stages and reduced the time of decolorization, delipidation, and RIM, such that even relatively thick tissues, like the 1.5 mm pig colon wall, could be effectively prepared for imaging in under 5 h. Overall, ADAPT-3D is simple to prepare, store, and use at room temperature, which are features that highlight strong potential for broad adaptability to fluorescence imaging.

ADAPT-3D maintains tissue integrity and fluorescent signal with partial delipidation in comparison to CUBIC

Since ADAPT-3D effectively rendered whole organs transparent while maintaining overall size, we next evaluated how decolorization and delipidation affected tissue integrity at macroscopic and microscopic scales. To benchmark the performance of ADAPT:PDL, which contains a chemical identified from the CUBIC chemical screen, we compared ADAPT-3D to the CUBIC protocol designed to preserve fluorescent reporters. After 12 hours of delipidation in CUBIC-L, a 1-mm thick brain slice (Fig. 2A, top) drastically swelled (Fig. 2A, middle), and upon washing with PBS, the cortex and hippocampus detached from the midbrain along the 3rd ventricle (Fig. 2A, bottom). Conversely, the balanced ADAPT:PDL preserved relative size of the brain slice over the same 12-hour time period (Fig. 2B, top-middle) and maintained structural integrity without tearing after washing (Fig. 2B, bottom). A similar detachment between the midbrain and cerebral cortex was observed in an adjacent section after 24 hours of CUBIC-L treatment (Supplemental Fig. 2A), while ADAPT:PDL still maintained tissue size at 24 hours (Supplemental Fig. 2B). Even the recommended CUBIC-L pretreatment at 50% dilution prepared in water before delipidation caused swelling and dislocation of the cerebellum on its own (Supplemental Fig. 2C), while the analogous ADAPT:DC step preserved tissue morphology (Supplemental Fig. 2D).

Both CUBIC-R+(M) and ADAPT-3D achieved refractive index matching in 1-mm brain slices to resolve at least 3.56 line pairs per mm; however, CUBIC-treated tissue was visibly enlarged (Fig. 2C). CUBIC is known to swell, a feature that can be advantageous in certain scenarios for increasing imaging resolution of the same tissue[20, 26]. Confocal imaging of the first in-focus plane revealed that brain slices in CUBIC-R+(M) were double the size of ADAPT-3D slices (Fig 2D, E). The fluorescent intensity of nuclei labeled with an ATTO-488 conjugated nanobody against histones was ten times brighter in the ADAPT-3D processed section compared to the CUBIC section when acquired with matched acquisition settings (Fig. 2D, F). These matched brain slices from contralateral hemispheres of the same brain were fixed with ADAPT-3D fixative to preserve endogenous fluorophores, yet the fluorescence intensity of endogenous tdTomato signal was nearly three times brighter in the ADAPT-3D processed sections (Fig. 2G). These differences were unexpected since N-methylnicotinamide in CUBIC-R+(M) was selected for its superior preservation of fluorescence relative to nicotinamide found in the basic CUBIC-R+(N) solution[35]. After digitally increasing the brightness of the CUBIC sample image, we observed that the ventricular space was distorted and lacked an intact choroid plexus (Fig. 2H) whereas ADAPT-3D maintained both ventricular architecture without swelling or collapse and an intact choroid plexus (Fig. 2I). Only ADAPT-3D tissue clearing enabled tracing of the choroid plexus in 3D from the lateral into the 3rd ventricle in a volumetric image acquired on the

confocal while CUBIC clearing failed to preserve the ventricular space and tore the choroid plexus (Video 1). Swelling during CUBIC delipidation also caused the thin leptomeningeal membrane lining the surface of the brain to break into fragments (Fig. 2J, Supplemental Fig. 2 E, F, top) while ADAPT-3D tissue processing preserved the continuous leptomeningeal layer (Fig. 2K, Supplemental Fig. 2 E, F, bottom). Thus, the preservation of tissue size by ADAPT-3D at all tissue processing steps and RIM maintains macroscale features for 3D visualization.

To minimize disruption of microscale features such as cellular membranes and intracellular compartments caused by excessive lipid removal, ADAPT:PDL was designed to partially delipidate tissues. Notably, after 3 hours of delipidation, ADAPT:PDL-treated sections retained the characteristic white appearance of lipid-rich fiber tracts following washing in PBS (Fig. 2L, bottom), whereas sections delipidated with CUBIC-L at 37°C degrees began to lose this contrast (Fig. 2L, top). By 24 hours, CUBIC-L treated sections exhibited partial transparency in the cortex (Supplemental Fig. 2A, bottom) consistent with extensive lipid removal, while ADAPT-3D processed sections remained relatively opaque and preserved white matter tracts (Supplemental Fig. 2B, bottom). These observations support that ADAPT-3D only partially delipidates brain tissue in contrast to CUBIC, which more aggressively removes lipids from myelinated and non-myelinated regions. Despite this gentler approach, ADAPT:RI effectively RI matched tissues even after only a partial removal of lipid for 3 hours (Fig. 1G).

To assess the effects of ADAPT-3D delipidation at a cellular level, we incubated fixed mouse peritoneal cells in ADAPT:PDL for 15 minutes and stained for lipids and intracellular compartments. Peritoneal cells from $LysM^{Cre}$;$Abca1^{fl/fl}Abcg1^{fl/fl}$ mice contain lipid droplets that stained positive for the neutral lipid dye LipidSpot (Fig. 2M, left), but after 15 minutes of incubation in ADAPT:PDL the macrophages no longer stained positive for lipid spot (Fig. 2M, right). However, the cell membrane still stained positive with the lipophilic dye $DilC_{18}(3)$, CellBrite Orange, that inserts into the lipid bilayer after 15 minutes of ADAPT:PDL treatment comparable to the membrane left untreated (Fig. 2N). Endosomes labeled with anti-EEA1 appear as punctate compartments throughout the cell (Fig. 2O, left), and are still retained following ADAPT:PDL treatment (Fig. 2O, right). Therefore, partial removal of lipid by ADAPT-3D also preserved cellular membranes and subcellular structures while removing certain light scattering lipids like those found in lipid droplets.

In conclusion, ADAPT-3D effectively cleared brain tissue for 3D imaging with a partial delipidating approach while preserving anatomical features and the intensity of fluorescent staining in comparison to CUBIC. ADAPT-3D and CUBIC both successfully lead to optically transparent tissues. However, only ADAPT-3D was able to maintain tissue size throughout all steps and avoid damage to the ventricular and leptomeningeal compartments. While CUBIC's optimized RIM solution is sufficient for visualization of endogenous and antibody-conjugated fluorophores under high laser power, ADAPT:RI preserves fluorescence intensity up to tenfold higher under identical imaging conditions. At a cellular level, ADAPT:PDL effectively removed neutral lipids while preserving membrane lipids and intracellular compartments. Tissue sections processed with ADAPT-3D still have clearly distinguishable white matter and remained opaque after delipidation unlike samples delipidated according to the CUBIC protocol.

Light-sheeting imaging with ADAPT-3D

ADAPT-3D effectively cleared whole brains and facilitated full-thickness confocal imaging in 1-mm slices. Thus we aimed to test its capacity to be coupled with light sheet

imaging of tissues bearing endogenous fluorophore reporter proteins. We applied the ADAPT-3D protocol to prepare the brain of a $ChAT^{Cre}$;$tdTomato^{fl/fl}$ mouse where cholinergic neurons were labeled with tdTomato[36] in a total of 4 days including 4 hours of RIM. The vasculature in this mouse, which was labeled with an intravenous injection of Dylight649-labeled Lycopersicon Esculentum lectin, was readily visualized into the core of the brain and highlighted the choroid plexus in the lateral ventricle (Fig. 3A, B; Video 2). The bright cell bodies of cholinergic neurons were observed throughout the cortex, as were the dimer axons that extended into fine dendrites observed in the first cortical layer (Fig. 3B). We also processed the brain from a CD11c-eYFP transgenic mouse [37] to test the ability of ADAPT-3D to preserve the more sensitive endogenous fluorophore eYFP [38, 39] during light sheet imaging of the whole brain (Fig. 3C). In some CD11c-$eYFP^+$ transgenic mice [37], we unexpectedly identified $eYFP^+$ cells with the morphology of dentate gyrus granule cells (Fig. 3D). This finding likely points to an off-target expression pattern in a mouse strain designed to study antigen-presenting dendritic cells of the immune system, but nonetheless nicely highlights the depth and preservation of eYFP that ADAPT-3D offers in whole mouse brain light-sheet imaging. Thus, in 5 days from tissue collection to imaging, ADAPT-3D methodology cleared the whole mouse brain for light-sheet microscopy with strong retention of the fluorescent intensities of endogenous reporter proteins eYFP and tdTomato.

A challenge for 3D imaging is the examination of bone with adjacent soft tissues such as in the skull-brain interface where reported vascularized skull channels exist through which immune cells can migrate into the dura mater [40-42]. Previous studies highlighted this challenge with use of popular methods like iDISCO, where the organic nature of the iDISCO solutions cause dehydration and consequent shrinking of soft tissue that tear the fragile meningeal space between the brain and skull, while simultaneously extinguishing endogenous fluorophores.[34] As a result, visualization of the leptomeningeal space including its skull channels has been limited. We sought to leverage the ability of ADAPT-3D to preserve both tissue size and endogenous fluorophores to visualize intact brain borders in a cleared whole mouse skull without the need for time-intensive antibody labeling. We decalcified the fixed intact brain and skull using EDTA with N-butyldiethanolamine, imidazole, and 1,2-hexanediol at pH 9.0 (Table 1; ADAPT:Decal) over a period of 3 days at room temperature while preserving endogenous fluorophores. Light sheet imaging of a whole skull from a $LYVE1^{CreER}$;$TdTomato^{fl/fl}$ mice injected i.v. with Lectin-Dylight649 cleared with ADAPT-3D captured the intact layers at the brain border without shrinkage (Fig 3E, Video 3). Visualization of the brain through the intact skull allowed identification of meningeal macrophages (Fig. 3F, arrows) and dural lymphatic vessels (Fig. 3F, arrowheads). In fact, there were distinctive $LYVE1^+$ channels bridging the skull and the leptomeningeal space, some of which were $Lectin^+$ and some that were not (Fig. 3G, asterisk; Video 3). The time from tissue acquisition and the initiation of processing to the time of light-sheet imaging with an intact skull was 8 days (1 day fixation, 2 days of decalcification, 2 days decolorization, 1 day of delipidation, 2 days RIM, such that light-sheet imaging was set up on the 8^{th} day). ADAPT-3D compared favorably with depth of clearing of brains with an intact skull, as it appeared to be faster from start to finish than the reported methods investigating light-sheet skull-brain imaging including iDISCO,[34] SHANEL,[5] or HYBRID.[5] A comparison of the total time frame used in ADAPT-3D relative to these other published processes is charted in Fig. 3H. Among other aqueous-based methods, CUBIC and its variants rendered marmoset brain hemispheres transparent in 29 days without attached skull.[35] We note that with the skull intact, and without the tissue shrinkage, vessels deeper in the brain were less well resolved in the time

frame we used (Fig. 3E-G), compared with our ability to resolve them well when the skull was removed (Fig. 3A, B). The absence of brain shrinkage with the ADAPT-3D method would naturally limit the direct exposure of the brain surface to clearing agents, likely requiring diffusion of clearing solutions through the skull bone in most areas, whereas methods that shrink the brain create a cavity within the skull for clearing agents to directly surround the brain, making it difficult to compare the methods directly. Nonetheless, ADAPT-3D stands out as a method that efficiently and successfully clears all layers of the intact brain borders while maintaining structural integrity.

Preserving fickle antigens with deep immunolabeling

Having observed that fluorescent reporter proteins are preserved with strong intensity, we next asked how immunolabeling was affected in the ADAPT-3D protocol. In particular, some antigens such as tight junctions are commonly masked with traditional fixation using 4% PFA at neutral pH, which have led some to seek alternative fixatives including those using methanol [43, 44]. Our modified fixative (Fig. 1A) combined with ADAPT-3D clearing enabled detection of claudin-11 and occludin tight junctional proteins along the arachnoid barrier and claudin-5- and occludin-expressing endothelial cells in the leptomeninges (Fig. 4A). Use of the modified methanol fixative in a side-by-side comparison revealed our approach was superior (Supplemental Figure 3).

As a further illustration of the utility of ADAPT-3D with immunolabeling protocols, we imaged full thickness preparations of the mouse ileum, staining for smooth muscle actin (SMA) and S100A9 in ileum of wildtype littermates (Fig. 3B, Video 4) and $TNF^{\Delta ARE}$ mice (Fig. 4C, Video 5) that develop transmural ileitis [45]. Widened, edematous villi and infiltrated neutrophils associated with ileitis in $TNF^{\Delta ARE}$ mice were evident (Fig. 4B, C; Videos 4, 5). ADAPT-3D was also used to visualize macrophage subpopulations differentially expressing CD163 and IBA1 in villi of the human intestine (Fig. 4D, Video 6). Villus height was readily measured, here averaging 344.6 ± 38.4 μm mean ± S.E.M. Across species, SMA was observed in conjunction with not only nuclei but also CD11c-eYFP cells in mouse, phalloidin in piglet, and $IBA1^{+}$ macrophages in the human (Video 7). In the human ileum, macrophages could be easily visualized throughout not only the villi but also concentrated populations particularly along the submucosal regions along the lower depths of the villi (Video 7).

Conclusions

In summary, we show that ADAPT-3D is a streamlined, efficient method for processing tissue, decalcifying bone and achieving RIM that holds numerous advantages for 3D tissue imaging. It is remarkably fast-acting compared with the reported tissue processing times of other aqueous methods. The use of compounds in a mixture and ratio that readily penetrates tissue likely underlies its fast action and ease of use. As we illustrate, ADAPT-3D is also designed to prevent morphological changes that result from dehydration, and is readily applicable toward optimal preservation of fluorescent reporters without the need to add compounds to "boost" these reporters. It also readily accommodates antibody staining. Finally, it holds advantage in the area of low toxicity. Recently, dichloromethane, a component in iDISCO+ and other related methods, was regulated by the environmental protection agency such that laboratory exposure will require monitoring (40 Code of Federal Regulations Part 751). ADAPT-3D does not use this regulated chemical, adding another attractive characteristic that should favor its adaptability for imaging applications in light microscopy. We suggest that this protocol will be of strong interest to pathologists and scientists interested in 3D fluorescence imaging.

Acknowledgments

This work was funded by NIH U01 AI163064, NIH R01 AI168044 and DP1DK130660 to GJR and the Imaging Core Component of NIH PO1AG078106 (JK, P01 PI; GJR, Core PI). DDL is funded in part by NIH T32HL007081. RC is supported by the Crohn's and Colitis Foundation (#938100). Additional support was provided by the Digestive Diseases Research Core Center at Washington University funded by NIH P30DK052574. We thank Ellen Schill and Rodney Newberry (WashU) for providing $ChAT^{Cre}$;$TdTomato^{fl/fl}$ mice. We thank John Long and Sylvia Morfant (Saint Louis University) for veterinarian experience with piglets, members of the Randolph laboratory for insight and feedback, and Mary Wohltmann for excellent technical assistance for mice. RC present address: Immunology Center of Georgia, Medical College of Georgia, Department of Physiology, Augusta University, Augusta, GA, USA.

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D.D.L conceptualized the study. D.D.L., K.A.T., L.C.D.S., D.L.D., R.R., R.S.C., C.G.H., and K.K. conducted experiments. A.K.J., B.H.Z., J.K., G.J.R. obtained regulatory compliance and funding. G.J.R. and B.H.Z. provided supervision. D.D.L., K.A.T., and G.J.R. wrote the manuscript draft with editing input from all authors.

Data Availability: Primary data are present within the manuscript. Raw image files in LIFF format can be made available upon request to GJR, BHZ, or DDL.

Declaration of Interests: Washington University and D.D.L., D.L.D., R.S.C., B.Z., and G.J.R. have filed a provisional patent on ADAPT-3D. ADAPT-3D solutions are being commercially developed by Leinco Technologies, Inc., Saint Louis, Missouri (https://www.leinco.com). J. K. is cofounder of Rho Bio that aims to develop therapeutics for the lymphatic vasculature. Other authors declare no competing interests.

TABLE 1. ADAPT-3D formulation

ADAPT-3D Refractive Index Matching Solution (ADAPT:RI)		
Reagent	*Amount*	*Final Concentration*
Iohexol	7.6 g	25.33% (w/v)
D-Sucrose	9.0 g	30% (w/v)
Urea	7.5 g	25% (w/v)
n-propyl gallate	0.150 g	0.5% (w/v)
1-thioglycerol	0.3 mL	1% (v/v)
Iodixanol	To 30 mL	
ADAPT-3D Decolorization (ADAPT:DC)		
Reagent	*Amount*	*Final Concentration*
CHAPS, ((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate	100 g	10%
N-methyldiethanolamine	250 mL	25%
N-butyldiethanolamine	50 mL	5%
1,2-hexanediol	100 mL	10%
Water	To 1L	
ADAPT-3D Partial Delipidation Buffer (ADAPT:PDL)		
Reagent	*Amount*	*Final Concentration*
Tetrahydrofuran	5 mL	50%
1,2-hexanediol	5 mL	10%
ADAPT-3D Decalcification Buffer (ADAPT:Decal)		
Reagent	*Amount*	*Final Concentration*
EDTA	150 g	15% (w/v)
Imidazole	150 g	15% (w/v)
N-methyldiethanolamine	100 mL	10% (v/v)
N-Butyldiethanolamine	50 mL	5% (v/v)
Water	To 1L	
ADAPT-3D Block-and-Stain Buffer (ADAPT:BS)		
Glycine	3.75 g	0.1 mM
Tween-20	0.833 mL	0.17%
Triton X-100	1.67 mL	0.33%
Donkey Serum	1%	5 mL
Alpaca Serum	1%	5 mL
BSA	1%	16.67 mL
H_2O_2	1.67 mL	0.1%
Dimethyl Sulfoxide	25 mL	5%
TBS	To 500 mL	
ADAPT-3D Fixative (ADAPT:Fix)		
Paraformaldehyde	4 g	4% (w/v)
Sodium Hydroxide (*Alternative: Triethanolamine)	pH to 9.0	
1X PBS	To 1L	
Sucrose		To 10% (w/v)

Methods

Mouse tissue

All mice were bred and housed in specific pathogen-free facilities at Washington University School of Medicine under standard housing conditions (12-hour light and dark cycles with feeding *ad libitum*). The Institutional Animal Care and Use Committee (IACUC) approved all experiments and procedures (protocol 22-0433). Animal experiments and the study were reported in accordance with the ARRIVE guidelines. All methods were performed in accordance with the relevant guidelines and regulations. Apart from CD11c-eYFP and CD11c-eYFP x Prox1^{CreER};tdTomato$^{fl/fl}$ mice, all other mice were on a C57BL/6 background. CD11c-eYFP mice [37] were a gift from Michel Nussenzweig (Rockefeller University). Prox1^{CreER} mice [46] were from Jackson Laboratory (Jax# 022075) and crossed to tdTomato$^{fl/fl}$ mice [47] from Jackson Laboratory (Jax #007909). ChAT^{Cre};tdTomato$^{fl/fl}$ reporter mice (Jax #028861), originating from a cross between were a gift from the Rodney Newberry laboratory at Washington University. LysMcre/+ (Jax #004781) were crossed to Abca1$^{fl/fl}$Abcg1$^{fl/fl}$ mice (Jax #021067) and to tdTomato$^{fl/fl}$ mice (Jax #007909). For tdTomato expression in inducible Cre mice, 12-week-old CD11c-eYFP x Prox1^{CreER};tdTomato$^{fl/fl}$ mice were treated with 2 mg tamoxifen (Sigma Aldrich, T5648), given by gavage and dissolved in sterile corn oil to 20 mg/mL for 3 total doses within a 7-day period. TNF$^{\Delta ARE/+}$ mice[45] were obtained in 2016 through the Cleveland Digestive Disease Research Core Center (NIH P30 DK097948) and continuously bred at Washington University. These mice were kept and bred as heterozygotes and always cohoused with wild-type littermates. Lyve1^{CreER};tdTomato$^{fl/fl}$ mice were generated by the Kipnis laboratory as described[48], and administered 2 mg tamoxifen by oral gavage 3 times over 1 week before euthanasia to process samples for imaging.

ADAPT-3D preparation and immunostaining

The following is a generalizable workflow for the processing of samples. We detail step-by-step for both sections and whole organs in Supplemental Protocol 1. However, the specific durations and volumes varied depending on the tissue type and size. In general, following fixation in modified ADAPT:Fix at 4°C ranging from 4 hours to overnight, all steps were performed at room temperature. To prepare ADAPT:Fix, 2 options were performed: (1) dissolving paraformaldehyde powder (Sigma-Aldrich, 158127) in PBS followed by adjusting pH to pH 9.0 with 10N NaOH; (2) adjusting commercially available paraformaldehyde solution (4% w/v) in PBS (sc-281692) to pH 9.0 with triethanolamine. Samples were rinsed twice in 1X phospho-buffered saline (PBS) containing 10 U/mL heparin and 0.3M glycine with at least 5 times of excess volume of the tissue. If bones are included, samples were immersed in excess volume of ADAPT:Decal at room temperature with daily change until soft to the touch. Hours after incubation with ADAPT:DC, samples become visibly partially transparent while incubation is generally performed for 6 hours every 1 mm of tissue; they were then washed in 1X PBS containing 10 U/mL heparin where visible transparency appears to reverse, which generally took about 1 hour at room temperature. For whole brain or brain slices, samples should be incubated with ADAPT:DC diluted into 0.2X PBS to limit swelling. Samples were incubated in ADAPT:PDL until some transparency (especially apparent for the brain) was observed followed by washing in 0.1X PBS for 30 minutes at room temperature until exchanged for 1X PBS containing 10 U/mL heparin. Finally, if just visualizing fluorescent reporter proteins, samples are immersed in ADAPT:RI until transparent. If immunolabeling was planned, samples were

incubated in ADAPT:BS with antibodies (see protocol below) rinsed with 1X PBS containing 10 U/mL heparin and 0.2% Tween-20, and then refractive index matched in ADAPT:RI until transparent. For most tissues, they were acclimatized in 0.5X ADAPT:RI solution diluted in 1X PBS for 30 minutes to 1 hour at room temperature before exchanging into 1X ADAPT:RI until transparent. The refractive index of ADAPT:RI was measured to be 1.50-1.51 by using a Digital Hand-held "Pocket" Refractometer (PAL-RI, ATAGO).

Comparison of ADAPT-3D to CUBIC

Whole brains from $LysM^{Cre}$;$tdTomato^{fl/fl}$ mice were fixed in 4% PFA (PH 9 10% (v/v) sucrose) for 24 hours, washed in 1X PBS containing 10 U/mL heparin (PBS-H), and then prepared into 1 mm sections using a vibratome. Matched sections from contralateral brain hemispheres of the same mouse were then cleared in a side-by-side comparison of ADAPT-3D or commercially available CUBIC reagents (TCI Chemicals) while varying the time of delipidation. ADAPT-3D samples were incubated in Decolorization buffer with 0.2x PBS for 6 hours while CUBIC samples were pretreated for 6 hours in 50% CUBIC-L diluted in water at room temperature. ADAPT-3D samples were washed with PBS for 3x30 minutes shaking, while CUBIC samples were switched directly into 100% CUBIC-L. Separate sections were delipidated for 0, 3, 12, or 24 hours in ADAPT-3D Delipidation buffer at room temperature or CUBIC-L at 37°C shaking. ADAPT-3D samples were rinsed for 2 hours in PBS with the first 30-minute wash in 0.1x PBS while CUBIC samples were washed for a total of 2 hours with three changeouts. All sections were labeled with anti-H2A-H2B (Histone-Label Atto488, Chromotek, tba488, 1:200) either in ADAPT-3D blocking buffer or in PBS with 0.5% Triton X-100 and 0.01% NaN_3 for CUBIC samples for 20 hours. Samples were washed overnight at room temperature in PBS-H with 0.2% (v/v) Tween-20 for ADAPT-3D treated samples or in PBS for CUBIC samples. CUBIC antibody labeling and washing was performed in accordance with basic CUBIC immunolabeling of 1.5-mm brain slices[18]. Samples were refractive indexed matched first for 1 hour in 50% ADAPT-3D diluted in PBS or 50% CUBIC-R+(M) diluted in MilliQ water and then incubated for 4 hours in their respective undiluted refractive index matching solutions before imaging. Fluorescent images were captured with a 10x lens (0.3 NA, Leica) in the first in-focus plane and then full thickness Z-stacks were acquired from the brain stem and cortex.

Mouse tissue immunostaining and imaging

For tight junctional protein staining, following euthanasia, C57BL/6J mice were perfused by transcardiac perfusion with PBS containing 10 U/mL heparin then by modified ADAPT-3D fixative. For preparation of mouse intestines, samples were fixed overnight in modified ADAPT-3D fixative, rinsed twice with 1X PBS containing 10 U/mL Heparin for 30 minutes each, incubated in Decolorization/Delipidation Buffer for at least 60 minutes, incubated overnight with antibodies against alpha smooth muscle actin-Cy3 (clone 1A4, 1:200, Sigma-Aldrich, C6198), Lyve1 (Abcam, ab14917, 1:300), S100A9 (Bio-techne R&D, AF2065, 1:400) in ADAPT-3D blocking buffer. Samples were rinsed in 1X PBS containing 10 U/mL and 0.2% (v/v) Tween-20 for 30 minutes at room temperature. They were then mounted in 0.5X ADAPT-3D Refractive Index Matching solution in 1X PBS for 30 minutes at room temperature before incubating in 1X ADAPT-3D Refractive Index Matching solution until transparent. For comparisons of preserving tight junctions in leptomeninges, samples were fixed with either a 4-h incubation with 100% methanol (wt/vol) or left in ADAPT-3D fixative. The following pre-treatments of ADAPT-3D methodology as described above were performed including decolorization and delipidation. For

immunolabeling, dorsal cortices were roughly dissected with a razor blade (0.5-1 mm thick) and stained with antibodies against Occludin (clone OC-3F10, Invitrogen, Cat. No., 331594, 1:200), Claudin-5 (clone 4C3C2, Invitrogen, Cat. No., 352588, 1:100), Claudin-11 (Invitrogen, Cat. No., 36-4500, 1:100) in ADAPT-3D blocking buffer. Samples were rinsed in 1X PBS containing 10 U/mL and 0.2% (v/v) Tween-20 at room temperature. Finally, cortices were mounted in 0.8 mm CoverWellTM imaging chambers containing refractive index matching solution.

Piglet tissue

Piglets (White Yorkshire x Landrace) were procured at 7 days of age and studied from an approved class A vendor (Oak Hill) then housed for 14 days before euthanasia. During this period, they were fed with Nutra-Start Liqui-Wean formula (Milk Specialties Global, Eden Prairie, MN) at Saint Louis University in accordance with approved IACUC protocol [2346, United States Department of Agriculture (USDA) registration is 43-R-011 to AKJ. Euthanasia was performed through an overdose injection of sodium pentobarbital. Tissue was then immediately collected and transferred to fixative in large buckets to allow for nearly 5 times the volume of tissue. Following fixation, tissues were washed with 1X PBS containing 0.3M glycine for 1 hour at room temperature, for a total of 3 changes. They were then stored at 4°C until proceeding with the protocol.

Human tissue preparation and immunostaining

Human intestinal tissue samples were collected according to and in compliance with IRB protocols #201111078 (to Rodney Newberry on behalf of the Digestive Diseases Research Core Center atPage 12/24Washington University) and #201111038 (to GJR) approved by the Washington University Human Research Protection Office / Institutional Review Board (HRPO / IRB). All experiments were performed in accordance with the relevant guidelines. Informed consent to acquire tissue from surgical or pathological waste was collected from each participant using protocol #2001111078. The research reported here was associated with tissue obtained under protocol # 201111038 that, as per these approved protocols, collects tissues procured under protocol # 201111078, using a waiver of consent. Accordingly, and in compliance with the associated regulations, no protected health information or identifiers were collected. No tissues were acquired from prisoners, fetuses, or pregnant women. For some intestinal samples, tissue was perfused with fixative directly into the ileocolic artery of the mesentery followed by immersion in fixative overnight at 4° C. 1 cm^3 samples were rinsed in 1X PBS containing 10 U/mL heparin for 2 hours, incubated in decolorization/delipidation buffer for 2 days, partial delipidation buffer for 1 day, followed by blocking buffer containing antibodies against CD163 (EDHu-1, Bio-Rad, 1:100), IBA1 (Fujifilm Wako, 019-19741, 1:100), and DAPI (Sigma-Aldrich, D9542, 1:200) for 2 days. Samples were rinsed in 1X PBS containing 10 U/mL Heparin and 0.2% Tween-20 for 2 hours and then incubated in refractive index matching solution until imaging.

Peritoneal cell isolation and imaging

Mouse peritoneal cells were collected through lavage of the peritoneal cavity with 5 mL of PBS supplemented with 5 mM EDTA. Cells were then centrifuged at 300xg for 5 minutes and resuspended in DMEM-F12 media containing (10% w/v FBS, 1x sodium pyruvate, 1X MEM Non-Essential Amino Acids Solution (100X, 11140050), 100 U/mL penicillin-streptomycin. Following resuspensions, cells were plated on 8-well chambered slides (ibidi, 80841) that were

manually coated with poly-L-lysine. After 2 hours of incubation, cells were fixed with 4% paraformaldehyde for 15 minutes on ice, permeabilized with ADAPT:BS, and incubated with a primary antibody against EEA1 (Cell Signaling Technology, C45B10) at 1:200 overnight at 4°C. On the following day, cells were washed with PBS containing 0.2% (v/v) Tween-20 for 5 minutes then incubated with Alexa Fluor® 647 AffiniPure-VHH® Fragment Alpaca Anti-Rabbit IgG (Jackson Laboratories, 611-604-215) at 1:400 dilution for 1 hour at room temperature. Following 2 washes with PBS containing 0.2% (v/v) Tween-20, cells were incubated with CellBrite Orange (1:200) for 30 minutes diluted in 1X PBS. After washing with PBS containing Tween-20, slides were mounted with FluoroBrite containing DAPI. Confocal microscopy was performed with an inverted Leica SP8 microscope that is equipped with 7 lasers with full spectral hybrid detectors (40X Objective lens, NA1.3, oil immersion) and set to pseudo-zoom by a factor of 7.5X.

Stereomicroscopy and confocal microscopy and tissue processing

Stereomicroscopy was performed using a Leica M205FA stereoscope equipped with a K8 digital color camera (2048 x 2048 pixels) at 12-bit for the acquisition of CD11c-eYFP-positive signal. Widefield microscopy was performed using a Zeiss Z1 examiner with an Objective LD SC Plan-Apochromat 20x/1.0 Corr M32 85mm equipped with a Colibri 7 light source. Confocal microscopy was performed with an inverted Leica SP8 microscope that is equipped with 7 lasers with full spectral hybrid detectors (20X Objective lens, NA0.75, oil immersions, or 25X Objective lens, NA0.95, water immersion). Alternatively, high-magnification images were acquired with the Stellaris TCS SP8 confocal microscope (Leica) using either a 10x objective (NA 0.4, Leica) with 2-2.5X digital zoom. In some cases, a USAF 1951 target was placed underneath the sample such that the resolution permitted by cleared tissue could be evaluated. The smallest element where all three black lines could be distinguished was used to calculate the resolution in line pairs per mm.

For comparison of visual transparency in organs like brain, spleen, and lung, stereomicroscopy was used. Following euthanasia with CO_2, 12-week-old C57BL/6J mice were perfused by transcardiac administration with 1X PBS containing 10 U/mL heparin (PBS-H) followed by either 4% PFA (pH 9.0) for ADAPT-3D or 4% PFA (pH 7.6) for iDISCO$^+$ brains and post-fixed overnight at 4°C. For ADAPT-3D processing of the lung and spleen, following fixation, they were washed in PBS-H for 30 minutes at room temperature and then incubated in ADAPT-3D Decolorization buffer for 48 hours. After washing in PBS-H for 30 minutes at room temperature, they were then incubated in ADAPT-3D delipidation buffer for 24 hours, washed in PBS-H again for 30 minutes at room temperature, and then incubated in RIM solution for 4 hours.

For clearing using iDISCO$^+$ methodology, brains were processed according to the protocol available at http://idisco.info with the only exception that the refractive index matching solution was ethyl cinnamate. Briefly, after overnight fixation, brains were dehydrated in graded methanol solutions starting at 20% v/v in MilliQ water for 1 hour at room temperature, progressing to 100% methanol, followed by 1 hour in chilled 100% methanol. The brains were delipidated with a 2:1 dichloromethane:methanol solution at room temperature and washed in 100% methanol two times and chilled at 4°C. Then, they were incubated overnight in chilled fresh 5% hydrogen peroxide in methanol at 4°C, rehydrated in a decreasing methanol series at room temperature until they were twice washed in 1X PBS containing 0.2% Triton X-100 for 1 hour at room temperature. Brains were again dehydrated in a progressive series of methanol into

a 2:1 dichloromethane:methanol solution when they were incubated for 3 hours at room temperature, twice washed in 100% dichloromethane for 15 minutes, then incubated in ethyl cinnamate for 4 hours. Following iDISCO^{+} or ADAPT-3D processing, samples were imaged with Leica M205 stereoscope that is mounted with DFC7000T camera. Areas were quantified by outlining brains at fixation or after refractive index matching.

Light-sheet microscopy and processing

Lyve1^{CreER};tdTomato$^{fl/fl}$ mice were injected retro-orbitally with a mixture of 40 micrograms of Lectin-Dylight649. After 5 minutes, mice were euthanized. Intact skull with brains were fixed overnight at 4°C, twice washed in 1X PBS containing 10 U/mL heparin (PBS-H) for 30 minutes each at room temperature, incubated in ADAPT-3D decalcification buffer with daily change for 3 days, washed in PBS-H once for 30 minutes at room temperature, and incubated in ADAPT-3D Decolorization buffer for approximately 48 hours at room temperature. They were washed in PBS-H once for 30 minutes at room temperature followed by incubation in ADAPT-3D delipidation buffer for approximately 36 hours, washed in PBS-H twice for 30 minutes at room temperature. Finally, they were incubated in 0.5X RIM in PBS to acclimatize the tissue for 1 hour at room temperature and then 1X RIM overnight.

For intact brains from CD11c-eYFP and ChAT^{Cre};tdTomato$^{fl/fl}$ reporter mice, following retro-orbital injection, the steps above were performed with the exception of decalcification. Following incubation in ADAPT-3D refractive index solution, samples were then placed in immersion oil (Cargille Labs, NA 1.52). Images were acquired on Miltenyi Ultra Microscope Blaze with a 4X/NA0.35 using 633 nm at 13% power with 40 millisecond exposure, 568 nm at 11% power with 20 millisecond exposure. Following acquisition, ome-tiff file formats were used to stitch in Stitchy™ (Translucence Biosystems) with default settings and exported as *.ims.

Image visualization and analysis

3D visualization was visualized on Imaris software (Bitplane Inc.) on v10.1.1 software. The area and fluorescence intensity of CUBIC and ADAPT-3D processed thick sections were measured using the mean gray intensity measurement function of imageJ software (v1.54p) after tracing the edge of the section for each replicate.

Statistical analysis

Data are presented as arithmetic mean ± standard deviation. For Fig. 1A, Shapiro-Wilk's test was performed to test for normality followed by a F-test for equal variances. Two-way ANOVA was performed to test the null hypothesis that there is no difference between mean intensities between pH or sucrose amounts followed by Tukey post-hoc test. p-values ≤ 0.05 were considered statistically significant. Experiments were repeated at least three times. A two-tailed, unpaired T-test was performed to compare CUBIC and ADAPT-3D sections.

Figure Legends.

Figure 1. ADAPT-3D tissue processing and refractive index matching render tissues optically transparent without shrinkage. (A) Comparison of fluorescent intensities of CD11c-eYFP follicles in Peyer's patches of mouse ileum following different fixative conditions (n = 4-5 follicles per condition, two-way ANOVA followed by Tukey test, *p-value ≤ 0.05, **p-value ≤0.01) and captured by stereomicroscopy. (B) Luminal side of fixed 600 μm thick mouse colons decolorized overnight (left) or left unprocessed (right) shown before (top) and after (bottom) a 60-minute incubation in ADAPT:RI. (C) Full thickness [1.5-2mm] cross sections of fixed human colon untreated (top) or incubated in ADAPT:RI for 10 minutes (bottom) without decolorization or delipidation. (D) Fixed mouse and piglet colon incubated for 60 minutes in ADAPT:DC, 60 minutes in ADAPT:PDL (left) and then incubated in ADAPT:RI for 30 or 60 minutes. (E) Fixed 1-mm brain slice from a $LysM^{Cre}$;$tdTomato^{fl/fl}$ mouse before ADAPT-3D processing. (F) The same section after 6 hours of ADAPT:DC followed by 5 hours of ADAPT:RI but without delipidation or (G) another section treated the same except for the addition of a 3-hour incubation in ADAPT:PDL before viewing immersed in ADAPT:RI. (H) Tiled confocal image of a single plane from a 1mm section with a $LysM^{Cre}$;$tdTomato^{fl/fl}$ reporter (red) and nuclei stained with anti-histone antibody (cyan). (I) Confocal acquired fluorescent z-stacks of the brain stem and (J) cerebral cortex from the 1mm section shown in F without ADAPT:PDL treatment (left) or from the 1mm section shown in G with 3 hours of ADAPT:PDL treatment (right). (K) Before and after top-down stereoscope image of fixed whole mouse brain and corresponding quantification of the brain area for duplicate samples following the iDISCO+ method including 4 hours of RIM in ethyl cinnamate or (L) following ADAPT-3D tissue processing consisting of 48 hours ADAPT:DC, 36 hours ADAPT:PDL, and 4 hours adapt RI. Dashed lines on graph in K and L link paired samples before and after. ** in J indicates the location of the corpus callosum.

Figure 2. ADAPT-3D maintains tissue integrity and fluorescence intensity for accurate 3D imaging without excessive lipid removal. (A) Stereoscope images of 1-mm fixed brain slice from a $LysM^{Cre}$;$TdTomato^{fl/fl}$ mouse before treatment (top), immediately after 12 hours in CUBIC-L at 37°C (middle), and after washing out of CUBIC-L buffer (bottom). (B) Matched brain slice from the contralateral hemisphere to that in A before treatment (top), captured immediately after 12hrs in ADAPT:PDL (middle), and after washing out of ADAPT:PDL (bottom). (C) Stereoscope image of CUBIC processed 1 mm section from A after immersion in CUBIC-R+(M) for 4 hours (left) and the ADAPT-3D processed 1mm section from B after immersion ADAPT:RI for 4 hours (right). (D) Tile scanned confocal image of the first in focus plane of the CUBIC processed section (left) and of the ADAPT-3D processed section (right) acquired with matched acquisition settings and displayed with equivalent scaling. (E) The area of CUBIC and ADAPT-3D processed sections measured from tiled confocal fluorescent images using imageJ software. (F) The mean fluorescence intensity of anti-Histone-atto488 and (G) of endogenous TdTomato in tiled confocal images acquired from sections processed with either CUBIC or ADAPT-3D (two-tailed unpaired t-test, standard error of mean bars, *p-value ≤ 0.05, *** p-value ≤ 0.001, ****p-value ≤0.0001). (H) Zoomed in view of the lateral and 3rd ventricle in the CUBIC processed section from D that was increased in brightness to be visible and (I) of the choroid plexus in the lateral ventricle of the ADAPT-3D processed section from D. (J) The leptomeninges on the cortical edge of a section processed with CUBIC vs (K) a section processed with ADAPT-3D. * in panel J indicates 1 of multiple sites of leptomeningeal tearing.

(L) Stereoscope image of white matter in matched sections where one was treated for 3 hours with CUBIC-L at 37°C (top) and the other with ADAPT:PDL for 3 hours (bottom) that were washed out of their respective delipidation buffers. (M) Fixed peritoneal fluid cells from $LysM^{cre}$;$Abca1^{fl/fl}Abcg1^{fl/fl}$ mice untreated (left) or incubated in ADAPT:PDL for 15 minutes (right) and stained with LipidSpot 610. (N) Representative image of CellBrite Orange staining for endosomes in fixed cells from peritoneal fluid without delipidation (left) or after a 15-minute incubation in ADAPT:PDL (right). (O) Representative image of EEA1 staining for endosomes in fixed cells from peritoneal fluid without delipidation (left) or after a 15-minute incubation in ADAPT:PDL (right) where combined endosomal and nuclear staining is shown as an inset.

Figure 3. Light sheet imaging of the whole mouse brain and of connections at the skull-brain interface visualized using endogenous fluorophores preserved by ADAPT-3D. (A) 3D whole-mount projection of the brain from a $ChAT^{Cre}$;$tdTomato^{fl/fl}$ mouse (white) injected retro-orbitally with Lectin-Dylight649 to label vasculature (fire) acquired by light sheet microscopy. (B) Extended display near lateral ventricle from A displaying blood vessels in the core of the brain and preservation of fine neuron dendrites in the cortex. (C) 3D whole-mount projection of a brain from a 16-week old mouse expressing CD11c-eYFP which was decolorized, delipidated, and incubated in ADAPT:RI followed by imaging with light sheet microscopy. (D) 500-micron coronal maximum intensity projection from whole brain of CD11c-eYFP where white arrow points to a CD11c-positive neuron. (E) A 50-micron x-y maximum intensity projection of the brain borders from a light sheet image of the whole skull from a $Lyve1^{CreER}$;$tdTomato^{fl/fl}$ (red) mouse injected i.v. with Lectin-Dylight649 and CD31-AF647 to label blood vessels (white). The layers of the brain borders are annotated with the following abbreviations: muscle [MS], skull [SK], dura mater [D], leptomeninges [LM], and brain parenchyma [B]. (F) Dorsal view of the light sheet imaging volume acquired from the whole skull in E where arrowheads point to dural lymphatics and full arrows point to meningeal macrophages. (G) Extended display of Lyve1 positive skull channels where the asterisk denotes consecutive skull channels bridging the skull bone marrow and meninges. (H) A graphical depiction of the relationship between the depth of light penetration in light sheet images of intact mouse skulls and the corresponding tissue preparation times reported for the different clearing protocols in the literature including the 8-day time frame determined here for ADAPT-3D.

Figure 4. Effect of ADAPT-3D on finicky antigens and compatibility with deep immunolabeling. A) Maximum intensity projections of tight junctions (arachnoid barrier: occludin in red and claudin-11 in green, endothelial-cell specific: claudin-5 in grey) found in leptomeninges from mouse imaged by confocal microscopy. B) Extended display showing en face and z-side projections of mouse ileum that was immunolabeled with alpha smooth muscle actin (yellow), lymphatic vasculature (LYVE-1, magenta), myeloid cells (S100A9, cyan), and nuclei (DAPI, grey) followed by imaging with confocal microscopy. C) Extended display showing en face and z-side projections of ileum from a 16-week-old mouse that expresses $Tnf^{\Delta ARE}$, a model of ileitis. D) Extended display showing en face, z-side, and 3D projections of fixed human ileum applied with decolorization, delipidation, immunolabeling with CD163 (green), IBA1 (red), and nuclei (DAPI, grey) followed by refractive index matching.

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