Tissue expansion of 'cleared' whole brains reduces contrast in *ex vivo* MRI

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Article

Keywords: ex vivo MRI, contrast, CLARITY, Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue hYdrogel, uDISCO, 3D imaging of solvent-cleared organs, ECi, Ethyl cinnamate Scale, whole brain, tissue-clearing, lipids, delipidation, tissue expansion, hyperhydration

Posted Date: September 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-877945/v1>

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Abstract

Multimodal imaging of optically-cleared brains, *ex vivo*, by Magnetic Resonance Imaging (MRI) and light microscopy (LM) presents unique opportunities for studying the brain at various scales and resolutions. However, CLARITY -cleared brains lack MRI contrast, implicating lipids as the major source of MRI contrast. We explored the *ex vivo* MRI compatibility of uDISCO, ECI and Scale -cleared brains. Surprisingly, uDISCO and ECI -cleared brains retain MRI contrast, whereas Scale-cleared brains show a severe loss of MRI contrast, as CLARITY. Determination of lipid-content in cleared samples shows that CLARITY, uDISCO and ECI are strongly delipidating, whereas Scale preserves most lipids. We conclude that MRI contrast can be associated with tissue expansion (and hyperhydration) rather than with lipid-content. Thus, we present two clearing methods compatible with *ex vivo* MRI.

Highlights

- We performed *ex vivo* MRI on CLARITY-, uDISCO-, ECI- and Scale cleared whole brains.
- MRI contrast is eliminated following CLARITY and Scale treatment, and preserved in hydrated uDISCO and ECI brains.
- We perform the first extensive lipidome analysis of CLARITY, uDISCO-, ECI- and Scale- cleared brains.
- CLARITY, uDISCO and ECI procedures are strongly delipidating, whereas the Scale clearing procedure preserves most of the lipids.
- MRI contrast can be obtained in delipidated brains, thus lipids alone are not a major source for MRI contrast.
- Tissue expansion and its consequent hyperhydration reduces MRI contrast.
- We present an approach to obtain *ex vivo* MR contrasting brain images following optical clearing.

Introduction

One rapidly evolving field of study is the study of the intricate brain connectome in three dimensions. However, the opaque nature of the brain and its large dimensions limit the use of high-resolution fluorescence light microscopy (LM) to image the brain in its entirety¹⁻⁴. This drawback has been addressed by emerging tissue-clearing techniques (e.g., CLARITY⁵⁻⁷) combined with advanced LM imaging methods, such as light-sheet microscopy⁸⁻¹⁰. Tissue clearing renders entire tissues transparent; allowing light to penetrate and traverse extended distances in the tissue^{7,11}, largely eliminating the need for tissue-sectioning and histology. However, clearing processes may produce structural deformities (e.g., anisotropic shrinkage or expansion)¹²⁻¹⁷ and these may complicate *post hoc* image-tiling and reconstruction of the entire tissue in 3D^{18,19}. Mesoscale imaging methods should, in practice, circumvent some of these hurdles. Indeed, modern fluorescence imaging is increasingly combined with complementary imaging methods and techniques for this purpose²⁰⁻²².

One attractive method is *ex vivo* Magnetic Resonance Imaging (*ex vivo* MRI)^{1,23}. *Ex vivo* MRI provide anatomical images of tissues (without the need to label) relatively fast (< minutes) and is non-damaging. Added benefits of *ex vivo* MRI is the ability to obtain images in higher resolution and signal-to-noise ratio than *in vivo* MRI²⁴. These make the method highly suitable for integration of data for precise image registration by, for instance, providing set-coordinates for correction of deformities and outlining major brain regions^{12,25–29}. Thus, multimodal imaging of cleared-tissues by LM and *ex-vivo* MRI should ease on the convergence between the micro- and mesoscale and aiding in framing the context of the fluorescence information^{1,30}.

Despite these advantages, dual LM-*ex vivo* MRI is not common (more common are *in vivo* MRI followed by *ex vivo* LM^{24,26,30}). In fact, it has been recently shown that several clearing methods, notably CLARITY, produce samples with no contrast when imaged by *ex vivo* MRI^{5,14,31–33}. These were interpreted to result from the removal of lipids (*delipidation*) from the tissues^{15,34–38}. This assumption implies that any clearing technique that involves delipidation (for instance, organic solvent-based clearing techniques DISCO^{39–43}, Ethyl-cinnamate- ECi^{44,45}, or others⁴⁶) should produce MRI-incompatible cleared brains, whereas tissues cleared by lipid-preserving methods, such as Sca/e⁴⁷ (or others^{15,34,48–55}) should retain contrast in *ex vivo* MRI. However, this binary classification remains unexplored, as but a handful of clearing techniques have been tested³⁴. Consequently, brain-clearing technique(s) combined with a highly detailed anatomic brain MR images have not been reported to date.

To address these unknowns, we systematically assessed *ex vivo* MRI contrast of transparent whole-brains cleared by various clearing techniques. In parallel, we examined the lipid content in cleared brains by two staining methods, Raman spectroscopy (RS) and quantitative lipidomics (by mass spectrometry; MS). Surprisingly, and in contrast to previous reports^{34,36}, we find that ECi and uDISCO—two highly delipidating methods (removing >80% and 99% of lipids, respectively)—preserve *ex vivo* MRI contrast following their hydration. On the contrary, images of CLARITY cleared whole brains (with >99% removal of lipids) are completely devoid of contrast. Counterintuitively, whereas Sca/e preserves >70% of lipids during the clearing process, it yields samples that are not MRI compatible, namely without any noticeable MRI contrast³⁴. Collectively, these show that lipid-content in cleared samples is not the major source for *ex vivo* MRI contrast. Curiously, we note that while CLARITY and Sca/e cause substantial expansion and hyperhydration of the brains, uDISCO and ECi induce shrinkage. These lead us to suggest that tissue expansion, and thus *de facto* increase in water content and decrease in the vicinity of water-interacting macromolecules, is the major cause for loss of MRI contrast. Together, we demonstrate two non-expanding and highly delipidating tissue clearing methods that are suitable for *ex vivo* MRI. These open new venues for multimodal imaging of large and transparent biological specimens by MRI and LM.

Results

MRI of cleared whole brains— We cleared whole brains of rats and mice by two solvent-based clearing methods, ECi and uDISCO, and by the aqueous-based clearing protocol CLARITY. ECi and uDISCO cleared

brains were amber-colored, translucent and of reduced size; with uDISCO cleared brains displaying stronger shrinkage (**Fig. 1 and Suppl. 1**). Inversely, CLARITY cleared brains swell significantly (>3 fold in volume) and were highly gelatinous and hyperhydrated (**Fig. 1, bottom**). These features were all previously described, demonstrating that our procedures achieve equal performances^{6,41,42,56}. We proceeded to image cleared brains by T1- and T2-weighted (T1W and T2W) MRI sequences. Rat and mouse control brains (i.e., PFA-fixed brains maintained in PBS) showed characteristic MRI contrast (**Fig. 1, control**) (e.g. ⁵⁷), whereas very weak MRI signal and contrast could be obtained when imaging the dehydrated uDISCO and ECI cleared brains (**Fig. 1 and Suppl. 1, ECI, uDISCO**). MRI signals were observed in discrete regions such as the ventricles and at the periphery of uDISCO or ECI cleared brains, which probably resulted from incomplete dehydration of the cerebral cavities. Blurred images were obtained when imaging ECI cleared brains, and this resulted from the samples' minute signals (**Figs. 1 and Suppl. 1**). On the other hand, and consistent with previous reports^{34,36}, CLARITY cleared brains showed very strong and homogenous MRI signal, though completely devoid of MRI contrast (**Figs. 1 and Suppl. 1; CLARITY**).

Our whole-brain MR images show a complete loss of *ex vivo* MRI contrast in CLARITY cleared brains. Similarly, uDISCO and ECI cleared brains yield no MRI contrast. However, unlike CLARITY, uDISCO and ECI cleared brains also lack any MRI signal. The latter is expected as uDISCO and ECI protocols are highly dehydrating. To conclude, chemically cleared brains show variable MRI signals, depending on their hydration state, however all three treatments abolish *ex vivo* MRI contrast.

Hydration of solvent cleared brains restores MRI contrast—We were curious as to whether we could obtain an MRI signal by re-hydration of solvent-based uDISCO and ECI cleared brains. We immersed uDISCO and ECI cleared brains in PBS for 72 hrs. This treatment reduced transparency and changed the color of the samples to opaque white. It was also complemented by visible expansion of the samples (**Figs. 2a, b and Suppl. 2**). Though initially undesired, as the brains are no longer optically clear, these transformations indicate proper water permeation into the samples, especially into deep regions of the tissue. Water entry into uDISCO and ECI cleared brains was somewhat surprising as these chemical processes significantly solidify the samples^{38,43,58}. Curiously, and although beyond the scope of this work, this observation demonstrates that hydrophilic-labeling strategies could be achieved after a full uDISCO and ECI clearing.

Hydration of uDISCO and ECI-brains significantly increased the MRI signals (**Figs. 2a and Suppl. 1**). Importantly—and unexpectedly—retrieval of the MRI signal was accompanied by restoration of the MRI contrast in uDISCO and ECI-brains (**Fig. 2a, 2c and Suppl. 1**). Various brain structures, such as corpus callosum, hippocampus, cortex, striatum, cerebellum, thalamus and lateral ventricles could be easily distinguished in MR-images of hydrated- uDISCO and ECI-brains (**Figs. 2a, Suppl. 3a**). Quantitative assessment of the contrast-to-noise ratio (CNR) of various brain regions showed that the MRI contrast in T1W and T2W images was significantly augmented; T1W images showing CNR values on par with those

of control brains (**Figs. 2c and Suppl. 3b, c**). We therefore examined different hydration durations (i.e., immersion in PBS up to 30 days after clearing) and find that maximal hydration and MRI contrast could be obtained following 3 days of hydration (**Suppl. 4**).

We were also interested in examining MRI contrast of hydrated uDISCO and ECi-brains in the presence of a high background signal (i.e., in PBS) compared to no background signal provided by the Fomblin Y (**Suppl. 5**). We find that T1W images showed a similar CNR when imaged in PBS compared to Fomblin Y, whereas CNR of T2W images were moderated possibly due to a limited greyscale range of the T2W images (**Suppl. 5**). We performed an analogous experiment using CLARITY cleared brains to see whether we could recover MRI contrast, as was previously suggested³⁷. We therefore immersed the CLARITY cleared brains in a refractive index (RI)-matching solution (2,2'-thiodiethanol; TDE), which shrank the sample^{49,59}, but failed to uncover MRI contrast (**Figs. 2a and Suppl. 1; CLARITY in TDE**).

Motivated by obtaining *ex vivo* MRI contrast for hydrated uDISCO and ECi brains, we speculated whether we could enhance it further by adding a contrast-agent. We locally injected one hemisphere of hydrated uDISCO and ECi brains with a Gd³⁺-based contrast agent (GBCA), whereas saline was injected to the other hemisphere as control. T1W images acquired a few minutes after GBCA injection showed an enhanced MRI signal and, more importantly, enhanced MRI contrast, exclusively at the site of injection (**Figs. 3 and Suppl. 6a**). Conversely, though GBCA in CLARITY cleared brains increased the, already extant, MRI signal at the injection site it did not reveal any MRI contrast (**Suppl. 6b**).

GBCAs enhance relaxivity of nearby water protons, producing signal intensity variances across the image. Thus, these results support our above observations that PBS immersion of uDISCO or ECi brains achieves sufficient hydration of the samples, which then translates into robust MRI signals. Moreover, whereas MRI contrast is absent in the hydrated (or TDE-immersed) CLARITY cleared brains, it is evident in hydrated uDISCO- and ECi brains. Lastly, GBCA can be readily used to enhance *ex vivo* MRI signal and contrast of hydrated uDISCO and ECi brains.

Electrophoretic tissue clearing of hydrated uDISCO and ECi brains abolish *ex vivo*-MRI contrast—Our results show that hydrated uDISCO and ECi brains maintain *ex vivo* MRI contrast, despite their anticipated delipidating-nature. This observation initially suggested to us that the remaining MRI contrast may be attributed to remaining lipids in the tissues, perhaps because uDISCO and ECi procedures are not as delipidating as CLARITY, as this has never been examined before. We therefore initially turned to try to remove more lipids from uDISCO and ECi cleared brains, as is achieved by CLARITY. The active lipid-removal step consists of placing the tissue within an electrophoretic tissue-clearing (ETC) system in a SDS-containing solution (ETC/SDS)^{60,61}. ETC/SDS caused uDISCO and ECi brains to expand, change color to opaque yellowish-white and turn soft to the touch; highly reminiscent of brains that underwent clearing via CLARITY (**Figs. 4 and Suppl. 7**). Notably, the change in color and features of the brain were mainly apparent at the outer layers of the brain, with noticeable “uncleared” interior. Concomitantly, when

imaged by MRI, we observed non-uniform reductions in MRI contrast, with the swollen outer layers showing pronounced reductions in MRI contrast the longer ETC/SDS was applied (seen as bright and swollen edges in the outer layers of the brain (**Fig. 4, arrowheads**)). ECi brains were more readily affected by ETC/SDS treatment than uDISCO brains, losing the MRI contrast in most of the tissue after prolonged ETC/SDS treatment (**Fig. 4**). Control brains (i.e., PFA-fixed brains without hydrogel embedding) showed the most severe loss in MRI contrast following prolonged ETC/SDS treatment (and were also highly fragile and deformed) (**Suppl. 7, control**). These results demonstrate the ability of ETC/SDS-treatment to abolish MRI contrast (but augment MRI signal) in the outer layers of the brains, especially in softer samples.

These observations raise two opposing hypotheses. The first states that solvent cleared ECi and uDISCO methods are not strongly delipidating and hence the prevailing *ex vivo* MRI contrast or, secondly, change in the tissue dimension (leading to hyperhydration of the tissue), rather than its lipid content is the reason behind loss of MRI contrast.

Assessing lipid content and composition in cleared brains—To address the first assumption, namely remaining lipids, we turned to compare lipid-content in cleared brains by various methods. Notably, these data are missing (or are very partial) for most clearing techniques^{36,62}. In fact, this missing data is behind the persistent debates regarding the extent of delipidation of each method (see discussion).

We aimed to assess the relative delipidation by staining cryosections of cleared brains with Oil Red O (ORO). ORO is a fat-soluble diazol-dye staining neutral lipids, lipid droplets and cholesteryl esters, but not biological membranes^{63,64}. We first validated the method by staining control and CLARITY- brains (**Suppl. 8**)⁶¹. Expectedly, cryosections from control brains showed positive ORO-staining, and staining could be reduced (i.e., destaining) by prolonged ETC/SDS treatment (**Suppl. 8; control-ETC**) and CLARITY-brains' cryosections showed very low ORO-staining (**Suppl. 8**). Unexpectedly, cryosections from uDISCO and ECi cleared brains showed extensive ORO-staining, even higher than control, which was amenable to destaining by subsequent ETC/SDS-treatment (**Suppl. 8; ECi-ETC and uDISCO-ETC**). These are somewhat consistent with the hypothesis that uDISCO and ECi may not be as delipidating as considered.

To test for phospholipid-content, we employed 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil). Dil is a lipophilic dye that intercalates into phospholipids, therefore is used to label cellular membranes⁶⁵ and, importantly, has been employed to assess lipids in cleared samples^{15,47,53,61,66}. We specifically employed a 'Dil washout assay' by staining thick (>3 mm) PFA-fixed mouse and rat coronal slices with a drop of Dil in each hemisphere, followed by clearing of the slices by different techniques (**Suppl. 9**). Indeed, Dil-stain was completely washed-out from CLARITY-brains (**Suppl. 9, slices 3B, 6B**). We next tested the various clearing steps from the uDISCO and ECi-procedures. Interestingly, we find that the dehydrating steps in uDISCO and ECi-protocols (*tert*-Butanol or ethanol + 2% Tween, respectively) are strongly delipidating, yielding significantly reduced Dil-staining; *tert*-Butanol

exhibiting the strongest Dil-destaining capacity (**Suppl. 9, slices 1, 2, 4, 5**). Moreover, the main delipidating reagents in uDISCO and ECI (i.e., Dichloromethane; DCM, and ECI, respectively) are also efficiently destaining, though the DCM solvent is stronger (**Suppl. 9, slice 7**). As a negative control, we similarly tested clearing by the Sca/e protocol⁴⁷. Sca/e achieves clearing by hyperhydration and therefore initially suggested to be non-delipidating⁵⁰ (though considered delipidating by others^{7,34}; see discussion). We find Sca/e to engender very gentle destaining of Dil from the tissue slices (**Suppl. 9, slices 3A, 6A**)⁵³. Together, our results suggest that CLARITY completely removes lipids, whereas uDISCO and ECI only remove phospholipids (**Suppl. 8 and 9**).

These observations motivated us to test a histochemical Dil-staining protocol on cleared brains cryosections (akin to the ORO-staining protocol). Surprisingly, when applied onto CLARITY, uDISCO or ECI cleared brains cryosections, the Dil-dye labeled all sections to a similar extent as control (**Suppl. 10, and see discussion**); inconsistent with our collection of results. These raised the possibilities that lipophilic dyes may be absorbed by the clearing reagents therefore dissuading us from drawing firm conclusions from cryosection labeling.

We therefore proceeded to analyze lipid-content by Raman spectroscopy (RS) that does not require any labeling. RS is used to measure composition and relative abundance of biomolecules, notably proteins and lipids, by their characteristic chemical bonds^{67–69}. Though lipids signatures span across the entire RS spectrum⁶⁹, we focused on the ratio between peaks appearing at 2850 cm^{-1} (representing CH_2 bonds of long aliphatic lipid chains) and 2940 cm^{-1} (attributed to CH_3 bonds as typically found in proteins) to assess protein/lipid ratio, as previously employed^{70,71}, including on a couple of cleared samples⁶².

We performed RS on slices excised from control and cleared brains. Spectra obtained from control brain slices showed the prototypical signature of biomolecules in the brain^{72,73}, with the expected lipids and proteins bands at 2850 and 2940 cm^{-1} , respectively (**Suppl. 11a; dashed region**). We noted that the 2850 and 2940 cm^{-1} peaks ratio was variable between different regions of the slice, however, with both peaks remaining highly visible (**Suppl. 11a, inset**)⁷². We analyzed CLARITY brain slices at two different time points: after partial (3 hrs ETC) or full clearing (9 hrs ETC), and observed progressive reduction in the $2940/2850\text{ cm}^{-1}$ ratio; indicative of the relative reduction in proteins compared to lipids (**Suppl. 11b**). Analysis of ECI and uDISCO cleared brain slices showed substantial contribution of the solvents to the spectra overlapping with the $2940/2850\text{ cm}^{-1}$ bands (**Suppl. 11c**). We extensively washed the cleared brains and slices in PBS and employed the unique peak of the solvent ($\sim 3060\text{ cm}^{-1}$) to assess its clearance. Though three days of washing caused a significant reduction in the 3060 cm^{-1} peak, the spectra remained marred with the solvents' signatures (**Suppl. 11d and e; red plots**). Nevertheless, in both instances, the lipid band was significantly reduced, indicating reduction in lipids compared to proteins (**Suppl. 11d and e; insets and black arrows**). Hydrophilic clearing reagents used for Sca/e did not overlap with $2940/2850\text{ cm}^{-1}$ band⁶². Sca/e cleared brain slices showed no reduction in lipids (**Suppl. 11f**). In order to compare the ratios between clearing techniques (and bypass potential differences that may arise

from acquisition from varying regions), we homogenized the brains. Homogenates from PBS washed-ECi and uDISCO brains showed a sharp attenuation in the 2850 cm^{-1} band, representing a strong decrease of lipids in the samples, vs. 2940 cm^{-1} (**Fig. 5a, magenta and green**), compared to control (**Fig 5a, blue**). Sca/e-homogenates did not show any reduction in the protein/lipid ratio (**Fig. 5a, red**). However—and interestingly—CLARITY homogenates displayed similar behavior as the CLARITY- treated brain slices, namely, exhibited a 2940/2850 cm^{-1} ratio of ~ 1 indicating a relatively larger reduction in proteins than lipids (**Fig. 5a, orange**).

Together, RS indicates a strong reduction in lipids following ECi and uDISCO treatment, but not following Sca/e. It also suggests that CLARITY may be deproteinating as it is delipidating. However, the relative-nature of the method, along with the overlapping spectra of the clearing solvents with the signatures of protein and lipids, may lead to inaccuracies in the quantitative assessment of lipids in cleared brains.

Owing to differences in the assessment of lipid-content by three different methods explored here (ORO, Dil and RS), we decided to proceed with mass-spectrometry lipidomics^{74,75}. Notably, despite the method's precision and resolution, it has not been previously applied on cleared specimens. We analyzed control and cleared homogenized mouse brains, in duplicates of 150 and 300 μg per sample (**Suppl. 12**). Controls yielded 53 nmol lipids/mg tissue, with Sca/e treated samples showing 38 nmol/mg lipids, thereby maintaining 72% of the lipids. CLARITY and uDISCO-samples showed almost a complete loss of all lipids in the brain (0.41 and 0.71 nmol/mg lipids, respectively), namely 0.78 and 1.3% of lipids remaining in the samples, respectively. ECi samples showed slightly higher lipid content, of 9.9 nmol/mg lipids representing less than 19% lipid-content compared to control samples (**Fig. 5b and Suppl. 12a**). Interestingly, both Sca/e and ECi treatments maintained the lipid composition as in control, with high proportion of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) (**Fig. 5b and Suppl. 12b**). Together, mass-spectrometry lipidomics support our results using the Dil-washout assay (**Supple. S9**) and RS (**Fig. 5a**), allowing us to conclude that CLARITY and uDISCO are equally and very strongly delipidating, closely followed by ECi, whereas Sca/e preserves most (>70%) of the lipids (**Suppl. 12b**).

We would like to emphasize that lipid-content (and lipo-typing) has not been systematically examined in various cleared samples. However, even when lipid-content was assessed, most reports employ a single method (e.g.,^{47,61,62}), resultantly, there are very few established methods for lipid assessment in cleared tissues. Our efforts show that different methods may yield varying and, at time, contrasting results. These suggest that lipophilic dyes may be absorbed by remaining clearing agents in the samples, thereby raising awareness towards the challenging use of lipophilic dyes in samples drenched in organic solvents. As systematical comparison between multiple clearing methods has not been reported before, we deem these demonstrations highly valuable.

Loss of MRI-contrast in a lipid preserving Sca/e- brain

Our results demonstrate that the MRI-contrast observed in ECI and uDISCO brains cannot be attributed to remaining lipids, as those are largely extracted (**Fig. 5a**). Therefore, we turned to corroborate our alternative hypothesis stating that loss of *ex vivo* MRI-contrast stems from tissue expansion and its subsequent hyperhydration (i.e., yielding a pronounced reduction in lipid/water ratio^{35,76}, rather than mere loss of lipids). Hyperhydration and non-delipidation are the two main features of the method *Scale*, thereby making it ideal to examine our hypothesis. We cleared PFA-fixed brains of mouse and rat by *Scale* for 20 and 90 days, and obtained clear and expanded samples of whitish color and gelatinous texture, as described (**Suppl. 13, PBS**)^{47,50}. The features are reminiscent of brains treated by the CLARITY protocol and by ETC/SDS (**Fig. 1, 4 and suppl. 7**). We also tested subsequent *Scale* clearing of hydrated uDISCO and ECI brains and observed that the tissues displayed similar changes in color and volume (**Suppl. 13, bottom**). *Ex vivo* MR-Imaging of *Scale cleared* brains yielded images with reduced MRI contrast, both in the non-treated PFA-fixed and in the hydrated ECI or uDISCO brains (**Fig. 6 and Suppl. 14, compare with Fig. 1, 2a, c**). Interestingly, T1W was more susceptible to loss in MRI-contrast than T2W images. Thus, these corroborate the hypothesis that lipids are not the major source of *ex vivo* MRI contrast, but rather the expansion of the tissue and excessive entry of water along reduction in lipid density and other water-interacting molecules in each voxel.

Discussion

The integration between different imaging modalities, notably LM and *ex vivo* MRI, is an attractive means for the development of preclinical and clinical diagnostic tools for large and intact biological specimens. This combination takes advantage of high spatial resolutions and signal specificity proffered by LM, with mesoscale-imaging of any tissue, at any depth, without the need of labeling select targets provided by MRI¹. This combination also provides unbiased means for image registrations, correction of tissue deformations, orientation within large tissues and validation of MRI signal by targeted fluorescent signals^{1,30}, to name a few (but also see^{12,15,25,61}). This endeavor, however, requires chemical clearing of the tissue^{7,11}, which has so far yielded samples without any *ex vivo* MRI contrast (**Fig. 1** and^{15,34,36,38}). These reports have implicated lipids as the major source for *ex vivo* MRI-contrast. This lipid-to-MRI contrast concept has gained attention and has even been suggested to provide means to assess extent of delipidation and tissue in-homogeneities by various clearing techniques^{15,34}.

Our initial aim was to explore whether other clearing techniques would be compatible with *ex vivo* MRI. Systematic analysis and modifications of two clearing techniques show that uDISCO and ECI methods are highly suitable for this endeavor, after these have been hydrated by PBS (**Fig. 2**). This was highly surprising as both uDISCO and ECI are suggested to be highly delipidating, much like CLARITY. The contrasting observations motivated us to systematically explore these discrepancies by using the conventional lipid dyes ORO and Dil to assess the relative lipid levels. However, these provided partially contrasting results: ORO-staining suggested that uDISCO and ECI cryosections retained neutral lipids, whereas washout of Dil-staining by clearing reagents and procedures showed these to robustly remove

phospholipids (**Suppls. 8-10**). Contrastingly, Dil-staining of cryosections prepared from post cleared tissues show no reductions in lipid levels by any of the clearing methods employed.

These results suggested to us that the presence of residual solvents in the cleared specimens may absorb the lipophilic dyes. Subsequent RS quantitative analysis corroborated this assumption, demonstrating the strong signatures of the solvents in ECI and uDISCO cleared brains, even after these have been extensively washed (**Suppl. 11**). Nevertheless, we eventually found (by comparing with our lipidomics assessment) that the Dil-washout assay gave the best indication regarding the delipidating-natures of uDISCO, ECI and CLARITY and the non-delipidating-nature of Sca/e (**Suppl. 9**).

Though the use of Dil-staining on cryosections of cleared specimens is not suitable for lipid quantification, it did provide some insights about the integrity of tissues treated by CLARITY, uDISCO and ECI (**Suppl. 10**). CLARITY cryosections showed very homogenous staining by Dil, with no defined brain regions. Furthermore, only CLARITY-cryosections appeared highly porous, whereas in ECI and uDISCO cryosections we did not see this sponge-like appearance and we could easily discern various regions in the sections, such as the hippocampus (**Suppl. 10**). This shows that CLARITY critically modifies the tissue and renders it highly porous.

We further tried to quantitate the relative content of lipids following clearing by RS. However, the solvents' signatures spanned the entire spectra; overlapping and overshadowing regions indicative of different lipids (**Suppl. 11a, c**)^{67,69}. We also observed regional variability in the protein/lipid signature. Homogenization of the samples helped bypass some of these challenges and allowed us to specifically focus on the high wavenumber region to quantify the relative abundance of CH₂ bonds of long aliphatic lipid chains and CH₃ bonds of proteins, as previously shown⁶². Although this analysis provides but a partial picture of the different lipids and fats in the tissue, it did show that uDISCO and ECI efficiently remove long aliphatic lipids, whereas Sca/e does not (**Fig. 5a**). Surprisingly, the results also suggest that CLARITY is relatively deproteinating (**Fig. 5a, orange**). Though the latter may appear at odds with measurements of phospholipid and protein content in CLARITY cleared brains^{33,36}, it is important to recall that RS measurements provide relative measurements rather than absolute amounts in the tissue, and that CLARITY is indeed deproteinating to various degrees^{5,7,77}.

To quantitate absolute amounts of all types of lipids in the cleared brains, we eventually performed mass-spectrometry-based lipidomics. Notably, this has never been demonstrated before. This method conclusively showed the strong delipidating-nature of CLARITY, uDISCO and ECI (removing 99%, 99% and 70% of the lipids, respectively) and, inversely, the lipid-preserving nature of Sca/e (removing < 20% of the lipids) (**Fig. 5b and Suppl. 12, Table 1**).

These findings resolve the debate regarding delipidation by Sca/e (⁴⁷ vs. ^{7,34}) but, more importantly, show no association between delipidation and *ex vivo* MRI contrast (**Fig. 7**). Strong support was obtained by *ex vivo* MRI of Sca/e cleared brains; showing very strong reductions in MRI contrast (>13-fold reduction in CNR compared to control; **Fig. 6, 7 and Suppl. 14**). In support, a recent study using the Clear^T method

which largely conserves tissue dimensions and lipids, preserves most MRI contrast³⁴. These conclusively demonstrate that lipids are not the major source of *ex vivo* MRI contrast.

What is the source for MRI contrast then? Our concerted observations raise an alternative hypothesis that expansion of the tissue is the primary reason for loss of *ex vivo* MRI contrast in cleared brains, which is a common feature in CLARITY, Scale, and ETC/SDS treatments (**Fig. 1, 4, 6, Suppl. 7, 13**). More specifically, MRI contrast of the brain is obtained by variations in the tissue's water-interacting molecules (including lipid)-to-water ratio (or myelin-to-water ratio^{35,76}). Expansion of the tissue increases the water density homogeneously across the tissue with a concomitant reduction in the density of water-interacting molecules, such as lipids (but not exclusively). Indeed, cryosections of CLARITY brains stained by Dil show a highly homogenous and porous tissue (**Suppl. 10**) and MRI scans of CLARITY cleared brain slices showed an increase in the diffusion rates and relaxation times of water molecules, resembling those of free water molecules³⁶. To try to mimic this effect, we continuously imaged water-absorbing gel beads (composed of super-absorbent polymers, such as the hydrogel) by MRI (**Suppl. 15a**). When placed in water and immediately imaged by MRI, each small bead produced a strong T2W image (**Suppl. 15b; i**). However, this signal progressively diminished as the beads expanded (**Suppl. 15b; vi**)—ultimately losing all MRI contrast until it was almost completely undetectable by MRI (**Suppl. 15c, dashed region**). Importantly, the beads did not lose any of their content during the process, rather simply underwent isotropic expansion and hyperhydration. We suggest this effect to represent brains undergoing expansion.

In conclusion, we show the feasibility of using cleared brains for *ex vivo* MRI, by uDISCO and ECI clearing techniques. First, it requires hydration of the specimens to reintroduce water molecules—the source for the MRI signal. Hydration of uDISCO and ECI produces non-transparent samples but, importantly, maintains their structure (**Fig. 2C**). Further, MRI contrast can be enhanced by addition of contrast-agents to the cleared samples. These capabilities suggest that this procedure should support multimodal imaging by initial LM-imaging of the transparent tissue followed by *ex vivo* MRI. We also suggest that multiple clearing techniques can be combined and that multiple rounds of LM imaging-hydration-MR imaging and clearing can be performed^{38,43}. This protocol should allow users to create atlases composed of registered MRI and LM images of entire tissues, as well as accelerate the development of novel multimodal agents by providing means to quickly validate MRI signals by fluorescence imaging¹. Lastly, we envision that this method could be implemented with LM to examine clinical histological specimens by *ex vivo* MRI to detect pathologies at various scales^{26,37}, analogous to pathology-optimized expansion microscopy^{20,78}.

Materials And Methods

Animals: We used 3-weeks to 6 months old, male and female, C57BL/6 mice and Sprague Dawley rats (Envigo, Israel). All animal procedure protocols were in accordance with the guidelines and regulations of the Technion, and were approved by the Animal Care and Use Committee of the Technion - Israel Institute

of Technology (Haifa, Israel, Ethic number IL-121-08-19). Animals were transcardially perfused with PBS (02-023-5A, Biological Industries) and neutral buffered 10% formalin (HT5011, Sigma-Aldrich) and the whole-brain was removed and immersed in formalin overnight, then washed and incubated in PBS at 4 °C.

Tissue clearing techniques; CLARITY: Procedure was performed using the X-CLARITY™ system and reagents (Logos Biosystems). Briefly, whole-brains were infused with hydrogel monomers at 4 °C overnight (C1310X, Logos Biosystems) and a subsequent hydrogel polymerization step was performed at 37 °C for 3 hrs at -90 kPa (C20001, Logos Biosystems). Polymerized brains were actively cleared in electrophoretic tissue clearing (ETC) solution containing 200 mM boric acid and 4% (wt/vol) SDS (pH 8.5) (C13001, Logos Biosystems) by the Tissue Clearing System (C10001, Logos Biosystems).

uDISCO: Whole brains were dehydrated by sequential immersion in incrementing concentrations of *tert*-Butanol (30, 50, 70, 80, 90, 96, and 100 % vol in DDW; 360538, Sigma-Aldrich) for 4-12 hrs in each solution with gentle shaking at 34 °C. Samples were delipidated in Dichloromethane (DCM; 270997, Sigma-Aldrich) for 1 h. The RI-matching solution mix included 1:2 benzyl alcohol and benzyl benzoate solution (BABB solution; 24122 and B6630, Sigma-Aldrich) added with DPE (67334, Sigma Aldrich) at 15:1 BABB to DPE ratio, and 0.4 % vol DL-alpha-tocopherol (Vitamin E; A17039, Alfa Aesar), as previously described⁴¹.

ECi: Whole brains were dehydrated by sequential immersion in incrementing concentrations of ethanol solutions (30, 50, 70, 100 % vol in DDW, 30, 50 and 70 % were adjusted to pH 9) supplemented with 2 % Tween-20 (P1379, Sigma-Aldrich) for 12-24 hrs in each solution with gentle shaking at 4 °C. Samples were delipidated in 100 % ECi (112372, Sigma-Aldrich) for at least 24 hrs until achieving transparency, as previously described⁴⁴.

Scale: Whole brains were immersed in Scale-A2 solution containing: 4 M urea (U5128, Sigma-Aldrich), 10 % (v/v) glycerol (191612, Sigma-Aldrich) and 0.1 % (v/v) Triton X-100 (X100, Sigma Aldrich) at pH 7.6 with gentle shaking at RT, as previously described⁴⁷.

Secondary treatments of cleared brains: hydration of ECi and uDISCO brains was achieved by immersion of the entire samples in PBS at 4 °C for 3 and 30 days. CLARITY brains were immersed in 100 % TDE overnight (**Fig. 2**). ETC/SDS of cleared brains was performed by transferring subsequently hydrated brains samples into the ETC chamber in ETC solution with the following settings: 1.2 A, 37 °C, for variable durations. For contrast agent injection, 1 µl of saline or 0.5 mM GBCA (Dotarem, Guerbet) was injected into one hemisphere of CLARITY- cleared and hydrated ECi and uDISCO brains, with NanoJectIII microinjector (Drummond Scientific) using glass capillaries (504949, WPI).

Magnetic Resonance Imaging: Images were acquired using a 9.4T MRI system (horizontal bore magnet); equipped with a cylindrical transmit volume coil (86 mm inner diameter) and a single channel surface coil (20 mm diameter) for detection (Bruker Biospec, Ettlingen, Germany). Brains were placed in cylindrical tubes and fully immersed in fomblin (fomblin-Y LVAC 06/6, 317926, Sigma-Aldrich) to minimize

susceptibility artifacts. MRI protocols included coronal and horizontal anatomical T2W and T1W scans. T2W images were acquired using a Rapid Acquisition with Relaxation Enhancement sequence (RARE), at 0.4- 0.6 mm slice thickness of 27-32 Slices, 100 μm in plane resolution with the following features: TR = 3500-4110 ms, TE = 36 ms, RARE factor = 12, FOV = $1.6 \times 1.6 \text{ mm}^2$ or $1.92 \times 1.92 / 2.2 \text{ mm}^2$, matrix size = 160×160 or $192 \times 192 / 220$, number of averages = 6. T1W images were acquired with a fast low angle shot (FLASH) sequence with the same geometry and resolution as the T2W images and with TR/TE = 320/4 ms, 30° pulse, number of averages = 6. For GBCA injections (**Fig. 3**), additional protocol of 3D T1W FLASH images were acquired, with the same geometry and resolution as before, and with TR/TE = 15/4.4 ms, 15° pulse, number of averages = 2.

Data processing was performed using Medical Image Processing Analysis, and Visualization (MIPAV) software (NIH). Regions of interest (ROIs) were manually selected and traced (**Fig. S3B**). The MRI regional contrast was calculated as the signal intensity difference with the thalamus. Signal was normalized to the background (i.e., noise) for quantifying the CNR. Intensity profiles were obtained by tracing a horizontal line over greyscaled images and plotted as intensity of voxel of interest (VOI) along trajectory of the line (i.e., distance).

Cryosection preparation: Control and cleared brains were sequentially immersed in 15, 20 and 30 % sucrose in PBS, for 12 hrs in each solution at 4°C . Brains were embedded in OCT and froze at -80°C . Tissue coronal sections of $30\text{-}\mu\text{m}$ thickness were cut on a cryostat and mounted on microscope slides (SuperFrost Plus, Thermo Scientific). To note, unwashed clear ECi and uDISCO brains disintegrated during cryosectioning, and the prolonged wash with sucrose in PBS facilitated the sectioning. Yet, CLARITY treated tissues were difficult to section smoothly at $<30\text{-}\mu\text{m}$, and washed-uDISCO treated tissues were more difficult to adhere to the slide.

Lipid staining: For ORO staining, slides were immersed in 0.3 % ORO in 60 % isopropanol (O0625, Sigma-Aldrich) and incubated 12 hrs at RT, then washed 3 times in PBS. For Dil staining of cryosections, slides were immersed in $5 \mu\text{g}/\text{ml}$ Dil (42364, Sigma Aldrich), for 2 hrs, then washed with PBS.

Slides were mounted (Immu-Mount, Thermo scientific) and scanned by an automated slide scanner (Pannoramic 250 Flash III, 3D Histech LTD). Histology (**Suppl. 8**) and fluorescent (**Suppl. 10**) images were converted to 32-bit greyscale, artifacts and background were excluded using Threshold tool (ImageJ, NIH), and intensity was calculated for each image. For relative ORO staining, the grey intensity of each slice was normalized to the averaged grey intensity in images from PBS control group, in each experiment.

Dil washout assay: A $1 \mu\text{l}$ from $1 \text{ mg}/\text{ml}$ Dil was dropped on each hemisphere of 3 mm thick coronal brain slices. Dehydration by *tert*-Butanol or ethanol + 2 % Tween-20 was stepwise for 4 days, as described above for uDISCO and ECi. The concomitant PBS control in the other hemisphere went through the same conditions of temperature, shaking rate and incubation time. CLARITY was after 3 hrs ETC, and Sca/e treatment was for 7 days at RT. DCM and ECi treatments were both for only 1 hr at RT.

Cleared brain homogenization: PFA-fixed mouse brains were weighted and cleared; Sca/e treatment was for two weeks on a chopped tissue, to facilitate the diffusion of Sca/e reagents. The CLARITY protocol was on a whole brain until it became completely clear. ECI and uDISCO cleared brains were extensively washed with PBS for 72 hrs. PBS was added to all the samples to reach 100 mg/ml (wet tissue weight before clearing). Samples were homogenized using a tissue homogenizer.

Mass-spectroscopy lipidomics: Two 30 µl samples from each homogenated brain, at 5 and 10 mg/ml, were taken for the lipidomic analysis. Lipid extraction was using a two-step chloroform/methanol procedure. Samples were spiked with lipid class-specific internal standards prior to extraction. After extraction, the organic phase was dried and resuspended in mass-spectroscopy acquisition mixture. The lipid extracts were subjected to mass spectrometric analysis, as previously described^{74,75,79,80}.

Raman spectroscopy: Raman spectra were acquired using an upright Confocal micro-Raman Microscope (LabRAM HR evolution, HORIBA scientific). Samples were illuminated by 532 nm laser (100 % laser intensity) through a 50× objective (LWD). The acquisition time was 10 s /point (except the spectrum in Fig. S11A that was acquired at 100 sec / point) with 3 accumulations. Brain homogenates (100 mg/ml), thick tissue slices excised from cleared and control brains, and a drop from ECI solution and uDISCO RI-matching solution mix were sampled on Quartz slides (042295, Alfa Aesar). For CLARITY slices at different ETC times, the same brain was sampled at 3 and 9 hrs ETC.

Declarations

Acknowledgements

Funding— Support was provided by the Israel Science Foundation (SB; 1096/17), Israel Innovation Authority, Kamin program (S.B., 66774) and the European Research Council (S.B., ERC-2019-STG). We would like to thank Dr Rachel Edrei for technical assistance with Raman Spectroscopy, Prof. Vidavsky Netta (BGU) and Prof. Tal Assaf (WIS) for comments and discussions. This work was performed in partial fulfilment of the requirements for the PhD degree of S.Olszakier.

Author contribution

These authors contributed equally: Shimrit Oz and Galit Saar.

SB and S.Oz designed the study, interpreted results and wrote the paper. S.Oz and GS performed and analyzed the experiments. S.Olszakier and RH performed preliminary experiments. MOK contributed to discussion.

Declaration of competing interest

The authors declare no conflict of interests.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

MRI of whole brains cleared by different techniques. Coronal and horizontal T2W and T1W MR-images of rat (left) and mouse (right) whole brains. Following fixation by PFA, brains were immersed in PBS (upper

panel) or treated by the ECi, uDISCO and CLARITY protocols (lower panels). Snapshots of brain samples, placed on a 0.5 cm grid paper, are shown in the rightmost columns. Images were taken before these were immersed in refractive index matching solutions, PBS, or Fomblin and before being scanned by MRI.

Figure 2

Hydration of ECi and uDISCO cleared brains enhanced MRI contrast. A. Rat and mouse brains were cleared by the ECi, uDISCO and CLARITY techniques, and hydrated by PBS immersion for 72 hrs (ECi and uDISCO) or immersed in TDE (CLARITY). Coronal and horizontal T1W and T2W images and a snapshot above 0.5 cm grid paper, are shown. B. Three brains from same aged mice (3-weeks) were treated by ECi and uDISCO protocols, or PBS (non-treated control). At the end of ECi and uDISCO protocols, the brain was cut in the midline and one hemisphere was hydrated with PBS for 72 hrs. The two hemispheres were re-assembled to demonstrate the change in volume following the treatments, and a snapshot on 0.5 cm grid paper was taken. C. CNR was calculated as the difference between cortex and thalamus MRI signal intensity, divided by the background signal (see Fig. S3C). CNR values of T1W and T2W coronal mouse (red) and rat (black) images are shown. Each point represents brain from one animal. Unpaired t-test was used to compare cleared with cleared+hydrated groups. One-way ANOVA following Dunnett test was used to compare treated groups with untreated (PBS) group in T1W or T2W protocol, significance is above each bar. ****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

Figure 3

GBCA injection enhanced MRI contrast of hydrate ECi cleared brain. Rat hydrated ECi brain was injected with 1 μ l of 0.5 mM GBCA to one hemisphere and saline to the other hemisphere. Coronal and horizontal (3D) T1W images revealed enhanced MRI signal and contrast at the GBCA-injection place. Intensity profile (white) shows the relative intensity along the VOI line graph (blue).

Figure 4

ETC applied on hydrated- ECi and uDISCO brains gradually reduced MRI contrast. Rat and mouse brains were treated with ETC after clearing by ECi and uDISCO protocols, for 6 and 24 hrs. Coronal and horizontal T1W and T2W MR- images and a snapshot on 0.5-cm grid are shown. White arrows demonstrate the progression of MRI contrast loss.

Figure 5

Quantitative lipid analysis in cleared brains. A. High wavenumber region of the Raman spectrum from non-treated (control) and cleared brain tissue homogenates. ECi and uDISCO samples were extensively washed in PBS before homogenization. B. Lipid amount and relative composition measured in non-treated (PBS) and cleared brain samples from 150 μg homogenated samples, by mass spectrometry analysis. PA - phosphatidate, PC - phosphatidylcholine, PE - phosphatidylethanolamine, PG - phosphatidylglycerol, PI - phosphatidylinositol, PS - phosphatidylserine, DAG - diacylglycerol, TAG - triacylglycerol, SM - sphingomyelin.

Figure 6

Scale treatment reduced MRI contrast. PBS (non-treated) and ECI cleared mouse brains were imaged after 20 and 90 days in Scale clearing reagents. Coronal T1W and T2W MR-images are shown (see also Fig. S14). CNR was calculated as in Fig. 2C.

Figure 7

The relation between lipid content, MRI contrast and volume change following clearing treatments. Schematic illustration of % lipids remaining after clearing (compared to non-treated control) obtained by lipidomic analysis, vs. the CNR obtained in MR images. The relative sample volume by the end of each clearing treatment is represented by the size of the circle.

Supplementary Files

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- [OzS.etal2021Figs.pdf](#)
- [Table1.xlsx](#)