Volumetric histological characterization of optic nerve degeneration using tissue clearing: literature review and practical study

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ABSTRACT

Tissue clearing technologies can greatly improve the depth and accuracy with which the three-dimensional structure of tissues, especially those of the nervous system, can be visualized. A review of the present literature suggests that the growing diversity and sophistication of various approaches have contributed to the expansion of this method to a greater variety of tissue types, experimental conditions, and imaging modalities. In the proof-of-concept study presented in this paper, a simplified and modified version of the tissue clearing method CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) was used in conjunction with fluorescent staining and immunohistochemistry to illustrate the three-dimensional structure and molecular characteristics of inflammatory and degenerative activity in the mouse optic nerve. Based on the studies summarized in this mini-review, and our impression from using the nCUBIC method, it appears that tissue clearing could be a viable approach revealing three-dimensional histological features of myelin-rich tissues under normal conditions and after injury.

Introduction

Achieving a complete understanding of the nervous system requires the accurate depiction of its morphology and organization. This necessitates volumetric imaging that captures information throughout large tissues such as whole nerves and whole brains, as opposed to traditional tissue sectioning methods that only allow information to be collected from one thin section at a time and destroy the three-dimensional (3D) structure of the tissue. A fundamental limitation on the depth and volume at which nervous tissues can be visualized is the ability of excitation light to penetrate deep into opaque tissues due to scattering of light as it moves through the tissue [1]. Light scattering results from interactions between incoming waves of light and molecules within the tissue, particularly lipids and large proteins, that deflect the light from its linear trajectory [2,3]. Light scattering is often described as arising from a mismatch of refractive indices (RIs) between different substances, where RI is a descriptor of the velocity at which light moves through each substance. Biological tissues are heterogenous structures containing many substances with differing RIs which are highly prone to light scattering. Thus, scientists have long sought to render tissues transparent by homogenizing the RI through lipid removal and/or dehydration to eliminate the inherent mismatch between low-RI aqueous/proteinaceous and high-RI lipidaceous cellular components [4].

Other methods involve the infusion of a high-RI solution to match the RI of lipids in the tissue [5], hyperhydration of the tissue to match the RI of protein domains [6], embedding of the tissue in hydrogel [7], or a combination of these mechanisms [8] (Table 1). While examples of these various approaches to tissue clearing are briefly described here, there have been several recent reviews that give a more comprehensive summary of the present literature and method development on tissue clearing [8,9,10].

Although tissue clearing efforts began in the early 20th century [11], the full potential of this approach was limited until the advent of optical sectioning-based microscopy techniques such as confocal and two-photon microscopy in the late 20th century. Optical sectioning allows for the collection of light from focal planes and the rejection of out-of-focus signal at depths throughout a sample. ‘Z-stacks’ of images collected from each focal plane within a sample can be aligned to reconstruct the 3D structure of the sample [12].
Table 1. Examples of tissue clearing methodologies.

| Method                | Lipid-solvating reagent | Refractive index matching reagent | Fluorescent protein activity | Compatibility with immunofluorescence assay | Effect on tissue morphology | Time to clear* | Notes | Reference |
|-----------------------|--------------------------|-----------------------------------|------------------------------|--------------------------------------------|----------------------------|----------------|-------|-----------|
| Solvent-based method  |                          |                                   |                              |                                            |                            |                |       |           |
| BABB                  | Methanol                 | Benzylbenzoate/ methylsalicylate  | Limited                      | Limited                                    | Shrinkage                  | 4 d            | Uses toxic reagents | Dodt et al. [13] |
| 3DISCO                | Tetrahydrofuran          | Dichloromethane/ dibenzylether    | Limited                      | Very limited                               | Shrinkage                  | 2 d            |                   | Ertürk et al. [4] |
| uDISCO                | tert-butanol             | Diphenylether                     | Yes                          | Limited                                    | Shrinkage                  | 3–4 d          | Maximizes shrinkage to allow imaging of large portions of tissue | Pan et al. [16] |
| iDISCO                | THF/Dichloromethane/SDS  | Dichloromethane/ dibenzylether    | Yes                          | Limited                                    | Shrinkage                  | 4 d            |                   | Renier et al. [14] |
| Simple Immersion      | 2,2-thiodiethanol        | sodium dodecyl sulphate (optional)| Limited                      | Yes                                        | Shrinkage                  | 1 wk           | Optional electrophoresis and hydrogel embedding | Staudt et al. [5] |
| TDE                   |                          |                                   |                              |                                            |                            |                |                   | Constantini et al., [28] |
| FRUIT                 | Fructose/urea/thioglycerol| None                              | Yes                          | No                                         | Minimal swelling            | 3 d            | Optional perfusion of solution | Hou et al. [19] |
| RIMS                  | Histodenz (D2158, Sigma- | None                              | Yes                          | Yes                                        | Swelling                   | 2 mo           | Commercial reagent can adjust RI of solution | Yang et al. [18] |
| FocusClear (FC-102, Taiwan, USA) | Dibutyltetc acid | Tween 20                          | Yes                          | Yes                                        | None                       | 1 d            | Commercial reagent | Moy et al. [29] |
| Hydrogel Embedding     | Isohexol/Diisocic Acid/ | SDS                               | Yes                          | Yes                                        | Minimal swelling            | 3 d            |                   | Park et al. [30] |
| SHIELD                | N-methyl-D-glucamine     |                                   |                              |                                            |                            |                |                   |                       |
| CLARITY               | SDS                      | FocusClear (FC-102, Taiwan, USA)/| Yes                          | Yes                                        | Minimal swelling            | 8 d            | Electrophoresis highly suggested | Chung et al. [7] |
| PACT                  | SDS                      | Histodenz (D2158, Sigma-Aldrich, USA) | Yes                          | Yes                                        | Minimal swelling            | 2 wk           |                   | Yang et al. [18] |
| PARS                  | SDS                      | Histodenz (D2158, Sigma-Aldrich, USA) | Yes                          | Yes                                        | None                       | 4 d            | Requires electrophoresis and perfusion of solution | Yang et al. [18] |
| Hyperhydration        | None                     | Fructose/glycerol                 | Yes                          | Yes                                        | Minimal swelling            | 3 d            |                   | Ke et al. [34] |
| SeeDB                 | Triton X-100             | Urea/glycerol                     | Yes                          | Yes                                        | Swelling                   | 4–6 d          | Time to clear depends on exact protocol | Hama et al. [20] |
| Scale                 | Triton X-100             | Urea/quintol                     | Yes                          | Yes                                        | Swelling                   | 2 wk           | Optional RI-matching step | Susaki et al. [6] |
| CUBIC                 | Triton X-100             | Urea/quintol                     | Yes                          | Yes                                        | None                       | 8 d            |                   | Kubota et al. [22] |
| CUBIC-cancer          | N-butyl/diethanolamine/ | Antipyrine/nicotinamide           | Yes                          | Yes                                        | None                       | 1 wk           |                   |                       |
| mCUBIC                | Triton X-100             | Urea/quintol                     | Yes                          | Yes                                        | None                       | 1 wk           | Time to clear is based on clearing of mouse optic nerve | This work |

*With the exception of mCUBIC, "Time to clear" is time to achieve sufficient optical transparency of thick sections; for mCUBIC, this value refers to the time taken to clear mouse ONs.
Building upon this innovation, Benzyl Alcohol/Benzyl Benzoate (BABB), one of the first modern tissue clearing techniques, was used to clear and visualize whole mouse brains [13]. Like earlier methods, this protocol consisted of tissue dehydration combined with immersion in other solvents to remove lipids and further homogenize the RI of the tissue. However, BABB was severely limited in that fluorescent labeling in BABB-cleared brains was only visible for several hours after the clearing/staining process due to the harsh dehydration process.

Another method, 3D Imaging of Solvent-Cleared Organs (3DISCO), partially addressed this limitation by replacing the BABB solution with less hydrophobic dibenzyl ether (DBE), which extended the visibility of fluorescent signals to several days in the mouse brain after 3DISCO clearing [4]. Immunolabeling-Enabled 3D Imaging of Solvent-Cleared Organs (iDISCO) provided further improvement in comparability with fluorescent labeling [14]. Tissue shrinkage and quenching of GFP signals resulting from tissue dehydration are drawbacks with dehydration-based methods such as DISCO [15]. Pan et al. [16] provided an additional improvement on 3DISCO in the form of a modified method, ultimate DISCO (uDISCO), which harnesses intense tissue shrinkage to image large portions of tissue, including entire organ systems. While this approach is not viable for applications where the absolute size and position of histological structures are important (such as exploring the borders and internal structure of cavernous degeneration in the murine optic nerve as presented in this paper), mCUBIC, unlike most other DISCO-based methods, supported the maintenance of endogenous fluorescent signals for months after immunolabeling and clearing.

These fundamental limitations to dehydration-based tissue clearing techniques led to the development of alternative RI homogenization methods that do not alter tissue morphology and maintain an aqueous environment to allow for fluorescent labeling. These include simple immersion techniques, in which the tissue is immersed in a high-RI solution for days to weeks to allow the solution to gradually diffuse into the tissue and homogenize the RI, possibly by breaking up large low-RI protein domains [8]. Several commercial clearing solutions such as FocusClear [17], Refractive Index Matching Solution (RIMS) [18], 2,2’-thiodiethanol (TDE) [3], and fructose-urea cocktail (FRUIT) [19] have been developed for simple immersion tissue clearing. While gentler than other tissue clearing methods, simple immersion techniques take a prohibitively long time to produce optically cleared tissue, especially in large samples such as the mouse brain [8]. Hydrogel embedding followed by lipid removal has been employed to achieve sufficient clearing in a shorter time.

These methods include Clear Lipid-exchanged Acrylamide-Hybridized Rigid Imaging/Immunostaining/In situ Hybridization-Compatible Tissue-hydrogel (CLARITY) [7], Passive CLARITY Technique (PACT), and perfusion-assisted agent release in situ (PARS) [18]. These techniques involve first embedding the tissue in a hydrogel by perfusing the animal with fixative, hydrogel monomers, and crosslinking molecules. Lipids are then removed passively by incubation in detergent and/or actively through electrophoresis. This is followed by immersion in a high-RI solution to further homogenize the RI [7].

In addition to the tissue clearing approaches mentioned above, some methods employ lipid removal using strong detergents in combination with urea-based hyperhydration of the tissue to reduce the RI of remaining molecular components and achieve efficient clearing while maintaining an aqueous environment that supports fluorophore activity. The first of these methods was Scafe, which uses a lipid-solvating detergent solution followed by immersion in a urea/glycerol solution that rehydrates the tissue [20]. Another hyperhydration-based tissue clearing method is Clear Unobstructed Brain Imaging Cocktails and Computational Analysis (CUBIC), which utilizes high levels of the detergent Triton X-100 to remove lipids from tissue and urea to hydrate the tissue. Lipid removal and hyperhydration are augmented by a sucrose-based RI homogenization step that promotes further clearing of the tissue [6]. Many modifications of the CUBIC method have been formulated since this initial protocol to improve CUBIC for various organs and whole organisms. There are several notable examples of this, including Tainaka et al. [21] who explored several variations of the CUBIC method that included various mechanisms including lipid and pigment removal, decalcification of bone, and RI matching. Kubota et al. [22] presented another modification of CUBIC, which employs lipid removal followed by RI matching in conjunction with a series of fluorescent markers to profile the shape and structure of cancer metastasis throughout entire rodent bodies. RI matching-induced tissue swelling was adequately compensated for, with the purpose of profiling cancer metastasis in whole bodies. However, the swelling followed by compensatory shrinking in this protocol makes it not ideal for experimental goals in which the exact position, dimensions, area, and borders of histological features are critical. Furthermore, RI matching may not be necessary for small, highly myelinated samples in which the main barrier to optical transparency is the high lipid content of the sample. This motivated our group to develop another modification of the original CUBIC method, referred to here as mCUBIC.
in which clearing of whole, small, highly myelinated samples (in this case, the optic nerve) is accomplished using delipidation as the sole mechanism to reduce light scattering.

Since mCUBIC does not disrupt the 3D structure of tissue and is appropriate for fluorescent labeling, it is suitable for answering experimental questions in which the precise shape and morphology of histological features are important. This led our group to apply mCUBIC to study degenerative activity in the optic nerve (ON) in mice that have undergone repeated mild traumatic brain injury (r-mTBI). This type of injury results in a bilateral cavernous lesion with inflammatory and degenerative activity in the ON proximal to the optic chiasm [23,24]. The structural nature of this lesion and the whole ON has been difficult to study using traditional methods because the damaged area extends beyond multiple planes of focus. Thus, as a proof-of-concept to demonstrate the usefulness of mCUBIC in investigating histological and dimensional characteristics of tissue damage, mCUBIC was used to clear whole ONs from mice that had undergone r-mTBI, sham mice subjected to anesthesia only (r-sham), and naive mice. To the best of our knowledge, this is the first description of microscopic imaging of the entire thickness of mouse ON with a sufficient amount of cellular detail after tissue clearing. Fluorescent markers were used to characterize structural and cellular aspects of the whole ON and r-mTBI-associated lesion. Confocal imaging was used on whole mCUBIC-cleared ONs to examine inflammatory and degenerative activity within the lesion area, including the novel visualization of abnormal F-actin clumping in the lesion. In addition, Z-stacks of images were used to generate quantifiable 3D representations of entire ONs and lesions. This pilot, proof-of-concept work was done to demonstrate the efficacy of modifications upon existing tissue clearing methods to explore diverse experimental questions and expand the use of tissue clearing to different tissue types and fluorescent labeling methods.

**Materials and methods**

mCUBIC, a modification of the existing tissue clearing method CUBIC, was used to render whole mouse ONs (n = 26) optically transparent to allow for the interrogation TBI-induced areas of damage using histological markers. This study was conducted using murine models and injury paradigms from unrelated ongoing experiments and is thus limited in scope.

All procedures were carried out in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research, the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Roskamp Institute’s Institutional Animal Care and Use Committee. Male, ages 13–23 weeks, httau mice expressing human tau on a C57BL/6 null murine tau background (005491, The Jackson Laboratory, ME) [25] were randomly assigned to either repeated mild traumatic brain injury (r-mTBI; n = 9) or repetitive sham (r-sham; anesthesia only; n = 11) groups [24,26]. R-mTBI mice received either one of two injury paradigms, producing a non-penetrating closed-head trauma i.e. hits, with no major neurological or anatomical abnormalities. In one paradigm, mice were subjected to two injuries per week for three months then optic nerves were extracted after three months, as established in Ojo et al. [27]. Under a different paradigm, mice were subjected to four hits in one day and ONs were harvested after 24 h. (unpublished data). Sham mice received anesthesia of equal duration and interval as the injured mice and optic nerves were extracted at the same time points. On a few occasions, naïve male httau mice (13–15 weeks) were used as well (n = 4).

**Tissue clearing**

In order to assure the flatness of the ON during imaging, the nerves were cut at the junction of the nerve and the eyeball immediately posterior to the optic chiasm. Prior to tissue clearing, whole nerves were fixed in 10% neutral buffered formalin (BBC-0141, BBC Biochemical, USA) at 4°C for 24 h and then transferred into 80% ethanol for long-term storage [24]. ONs were then severed at the junction between the nerve and the globe of the eye in order to reduce technical artefacts associated with keeping the intraretinal portion of the ON intact. Fixed ONs were then treated with a homogenization solution composed of 25 w % urea (29700, ThermoFisher Scientific, USA), 25 w % N,N,N',N'-Tetrakis(2-Hydroxypropyl)ethylenediamine (122262, Sigma-Aldrich, USA), and 15 w % (Triton-X 100 (9002–93-1), Sigma-Aldrich). This solution is identical to the lipid-clearing reagent used in the original CUBIC protocol produced by Susaki et al. (Figure 1 and Table 1) [6]. Each nerve was immersed in 3 ml of this solution at 37°C with gentle shaking for 3 d, after which the solution was changed, and the nerves were immersed in fresh solution for an additional 3 d. The solution was exchanged a second time and the nerves remained immersed for an additional 3 d. The cleared optic nerves were washed in phosphate buffered saline (PBS pH 7.4; 20X, D1306, Invitrogen, USA) at room temperature (RT) for 90 min with two PBS changes at 30 min intervals prior to the immunohistochemistry assay.
DAPI and TRITC-phalloidin staining

Following mCUBIC treatment and two washes in PBS, nerves were immersed in a 1:500 solution of DAPI (D1306, Invitrogen) diluted in PBS and “Super Sensitive Wash Buffer (20X) (HK583-5 K, BioGenex, USA) for 15 min. The nerves were given two additional washes in PBS. A subset of nerves was then treated with TRITC-based Vectashield HardSet Antifade Mounting Medium containing Phalloidin-Rhodamine to counterstain actin filaments (H-1600, Vector Laboratories, USA) for 15 min. These nerves were then washed twice in PBS and returned to clearing solution for storage at 4°C until imaging.

Immunofluorescence assay

A subset of nerves was subjected to an immunofluorescence assay to label anti-glia fibrillary acidic protein (GFAP), a protein expressed in astrocytes and other cell types in the nervous system [31]. Following removal from clearing solution and three 30 min washes in PBS the nerves were treated for 1 h at RT in 3 ml R.T.U. Animal Free Blocker and Diluent (SP-5035-100, Vector Laboratories) prepared according to the manufacturer protocol. The ONs were then incubated overnight at RT with rabbit anti-GFAP polyclonal antibody (IgG, PA1-10019, AB_1074611, Invitrogen) diluted 1:1000 (1 ug/ml) in PBS in 3 ml volumes. After three washes in PBS for 30 min per wash, nerves were treated with goat anti-rabbit IgG H&L Alexa Fluor 488 (ab150077, Abcam, UK) diluted 1:500 (1 ug/ml) in PBS for 1 h at 37°C. The ONs then underwent three 30 min washes in PBS and were immersed in DAPI (D1306, Invitrogen) diluted 1:500 (1 ug/ml) in PBS in 3 ml volumes for 15 min, followed by immersion in Super Sensitive Wash Buffer (20X; HK583-5K, BioGenex) for 15 min at RT. After two additional washes in PBS for 30 min per wash, the nerves were stored in clearing solution until imaging. One nerve was subjected to clearing without exposure to primary antibody to serve as a negative control. Immediately prior to imaging, nerves were placed on a glass slide in a 200-µm deep well created with slide spacers (654002, Grace Bio Labs, USA), immersed in fresh clearing solution, and covered with a coverslip pressing against the nerve. This preparation was allowed to sit for 20 min prior to imaging.

Imaging and data analysis

Tiled mosaic images of consecutive Z-slices spanning each nerve were taken with a 10x objective using a ZEISS LSM 800 confocal microscope (Carl Zeiss Microscopy, USA). In addition, areas of interest were imaged with 20x and/or 63x objectives. The images were initially reviewed using Zen 2.3 software (Carl Zeiss Microscopy) and then exported to ImageJ [32] as tagged image file format (TIFF) files for further analysis. For 3D visualization and modeling of image stacks, longitudinal slices of ONs were imported into 3D software Mimics Innovation Suite v.22 (Materialise USA). Nuclei were isolated using manual thresholding. For the ON
stained with Phalloidin-Rhodamine (TRITC), the borders of the lesion (shown in red; Supplementary Video 2) were manually isolated. Volumetric measurements of the lesion and surrounding nervous tissue were also collected. The resultant three-dimensional models were used for further visualization in 3DS Max 2018 (Autodesk, Inc., USA).

Results
ONs achieved transparency sufficient for volumetric imaging within 6–10 days. The chiasm and region of the optic nerve within 100 µm of the anterior border of the chiasm were the last to become transparent while clearing of the distal portions of the nerve and took approximately 4–6 days. It was observed that samples immediately began losing transparency upon removal from the clearing solution. Transparency could be restored by re-immersing ONs in clearing solution with shaking for several hours to several days, depending on the size of the tissue and the extent of opacity. It was possible to restore tissue transparency even in the presence of urea crystallization, which occurred after 6–8 weeks of tissue storage in clearing solution at RT. Fluorescent signals were still visible in ONs after several months of storage in clearing solution in a dark environment.

mCUBIC + DAPI stain of cell nuclei in the lesion area
Approximately 24 h after mice were subjected to four injuries per one day, mid-depth confocal imaging of DAPI-stained nuclei revealed well-defined bilateral lesions within the body of each ON near the chiasm, characterized by a high concentration of DAPI-stained cell nuclei (Figure 2). Imaging throughout the entire Z-plane of a DAPI-stained naïve nerve from an age-matched mouse enabled 3D reconstruction of the entire ON revealing well-preserved and uniform nuclear distribution throughout the entire length of the tissue (Supplementary Video 1).

mCUBIC + GFAP + DAPI staining analysis of inflammatory activity in the mouse whole ON
The immunofluorescence assay for GFAP counterstained with DAPI revealed that GFAP expression, a marker of astrocyte activity, was detectable at various depths in mCUBIC-cleared tissue. Some nonspecific background staining was present, likely due to aldehyde-induced autofluorescence. This was partially corrected by using a median filter in ImageJ but did not chemically block aldehyde-induced autofluorescence. In mice that were subject to an injury paradigm consisting of two hits per week for three months, high magnification imaging of the lesion area stained 24 h after last injury revealed a combination of 1) GFAP-negative cells, 2) DAPI-stained nuclei, and 3) GFAP positive cells containing DAPI stained nuclei (Figure 3). There was a high concentration of GFAP-positive astrocyte processes within the lesion area as compared to non-lesioned areas and naïve tissue.

mCUBIC + GFAP for 3D reconstruction of ON size and shape
Moreover, the immunofluorescence assay for GFAP of a whole r-sham mCUBIC-cleared ON enabled the

Figure 2. Optic nerve (ON) from 15-week-old htau mouse 24 hours after four hits (r-mTBI) per day. (a) Longitudinal section at mid-depth in ON. Nuclei are stained blue with DAPI. Scale bar = 100 µm. (b) Inset shows the area of increased cellularity in lesion at higher magnification. (c) Inset shows an area of tissue outside the lesion area at higher magnification. Inset scale bars = 50 µm.
collection of confocal Z-stacks throughout the entire volume of tissue that were used to create detailed 3D reconstructions of the ON. Transverse sections of this reconstruction were well defined by ellipses (Solidity shape descriptor >0.95) and of sufficient quality to quantify the size and structure of the nerve at various points (Figure 4). Expression of GFAP was more effective for this type of cross-sectional analysis compared to DAPI only staining (Figure 2). This is because GFAP expression is uniform throughout entire cells, while
Figure 4. Confocal images of longitudinal optical sections of an r-sham optic nerve from 79-week-old htau mouse 6 months after sham treatment. Immunofluorescence assay for GFAP. (a) a mid-plane image of the optic nerve. White lines indicate the placement of the transverse sections (generated by the Reslice command in ImageJ) along the length of the nerve. (b) Most posterior transverse section; (c) middle transverse section; (d) anterior transverse section. An ellipse was fitted (Fit Ellipse) around perimeter of each transverse sections using the Oval Selection Tool in ImageJ. Longitudinal section Scale bar = 500 µm; Horizontal and vertical diameters for the three elliptical transverse sections were measured at (b): 491 µm (horizontal arrow) and 277 µm (vertical arrow) (c): 502 µm (horizontal arrow) and 272 µm (vertical arrow), and (d): 373 µm (horizontal arrow) and 233 µm. Transverse (oval) section scale bar = 100 µm.

Discussion

A wealth of tissue clearing methods has provided an array of mechanistic approaches to optically clearing tissue. These efforts have successfully expanded tissue clearing-based approaches to suit a wide variety of sample types and experimental goals. In the present work, mCUBIC, a simplified version of the previously published tissue clearing method CUBIC [6], was used to clear whole, NBF fixed mouse ONs and to image the tissue structures throughout the thickness in considerable cellular detail. Although the original CUBIC method, the dehydration and shrinkage-based method uDISCO [16], the aqueous clearing method SeeDB [34,35] have all been used to render whole mouse ONs optically transparent, microscopic imaging of ONs was not performed in these studies. iDISCO [36], tetrahydrofuran (THF) and BABB [37], and hexane [38] have been used to assess axonal pathfinding in whole mouse ONs, but these studies relied on visualization of axonal tracing rather than immunostaining. The present proof-of-concept study is the first application of tissue clearing to the whole ON where macroscopic morphological structure, molecular structure, and protein expression patterns were simultaneously assessed in the same tissue using volumetric imaging of immunostained and fluorescently stained ONs. In particular, our study revealed a higher concentration of cell nuclei in the lesion area (Figure 2), a known characteristic of r-mTBI-induced ON lesions [24]. We observed a high concentration of GFAP-positive astrocyte processes within the lesion area, which is consistent with the known association between...
increased astrocyte activity and inflammatory activity in nervous tissue [39]. Surprisingly, we found that previously unobserved abnormal clumping of F-actin stained with Phalloidin-Rhodamine in the lesion area is also a characteristic of mouse ONs subjected to r-mTBI.

The development of mCUBIC, a relatively simple and inexpensive method, was largely driven by previously observed limitations of the original CUBIC protocol’s ability to preserve tissue morphology. These factors were particularly confounding to our experimental aims, which involved using labeling of proteins to measure the precise 3D structure of lesions of inflammatory activity induced by r-mTBI. Our impression is that mCUBIC not only preserves tissue morphology, but it is also compatible with the use of standard fluorophores. This represents an improvement upon many previous tissue clearing methods, in which immersion in RI-homogenizing solutions causes considerable tissue swelling [20] and/or tissue dehydration when using harsh solvents quenches fluorescent signals [13]. Therefore, compared to other tissue clearing methods, mCUBIC may be particularly suited for experiments based on fluorescent labeling where undistorted tissue

Figure 5. Confocal imaging of longitudinal optical sections of optic nerve of a 13-week-old mouse at 24 hours after r-mTBI consisting of four hits for one day. The images were collected from a vertical Z stack of images starting at (a) top of nerve and images (b – j) progressing downwards in depth. Five consecutive images were averaged in ImageJ to generate one image in each panel. Nuclei are stained with DAPI (blue) and F-actin stained by a Phalloidin-Rhodamine (red-orange). Scale bar = 500 µm. Depth of imaging in each panel is as follows: (a) 0–24 µm, (b) 25–48 µm, (c) 49–73 µm, (d) 74–97 µm, (e) 98–121 µm, (f) 122–145 µm, (g) 150–169 µm, (h) 174–194 µm, (i) 198–223 µm, and (j) 223–242 µm.
morphology and structure are important. Because lipid removal is the primary mechanism of mCUBIC clearing, this method may be ideal for small, lipid-dense samples such as the ON.

There were several logistical issues that became apparent when implementing mCUBIC, many of which are universal to all tissue clearing approaches. One of these issues was the large amount of time needed for volumetric imaging of the entire ON using confocal microscopy. The speed of imaging in this case is limited by the necessity of raster scanning across the entire sample at each thin plane of focus throughout the entire depth of the tissue. The time taken to move the excitation laser to capture information from the entire volume of tissue adds up to many hours of data acquisition. Some researchers have addressed this problem using light sheet fluorescent microscopy, which allows for much faster imaging using a larger area of excitation and resolution comparable to other common microscopy methods \cite{37,40}. An additional challenge was the large amount of data storage needed for large Z-stacks of images representing the entire ON, often several gigabytes (GB) in size. External hard drives were sufficient for storing and transporting these data. Common free-of-charge image processing softwares, such as ImageJ and Zen Lite, have limited volumetric image rendering capabilities and had difficulty processing large files generated from cleared tissue using a standard office-type desktop computer (16 GB RAM). Image processing programs with more sophisticated volumetric image rendering functionality do exist, but their cost is prohibitive to many laboratories. All technical issues with data acquisition, storage, and analysis will resolve as the fields of microscopy, file storage, and 3D image rendering catch up to the needs of volumetric imaging. Ultimately, our results using mCUBIC for clearing and histological analysis of the mouse ON supports the notion that simplifications and modification of existing tissue clearing protocols, as well as the emergence of novel tissue clearing methods, will continue to improve the quality of sample obtained from tissue clearing, increase the depth at which tissue can be imaged and fluorescent signals can be detected, and expand the body of experimental approaches and sample types for which tissue clearing is useful.

Conclusions

This paper summarizes a proof-of-concept study employing a simplified and streamlined version of an existing tissue clearing method. This modified protocol, mCUBIC, is presented in the context of a brief literature review on the breadth of tissue clearing methods and their various applications. mCUBIC’s potential for exploring the 3D structure of inflammatory and degenerative activity in whole mouse ONs is illustrated using immunohistochemistry and fluorescent stains. Confocal imaging mCUBIC-cleared ONs revealed lesions of inflammation that are known to occur in our r-mTBI model, as characterized by increased DAPI-stained nucleus density, increased GFAP-reactive astrocyte processes, and abnormal F-actin clumping in the lesion area. The borders and volume of the lesion and entire nerve were assessed using 3D reconstruction of volumetric images, providing a reliable measure of the extent of cavernous degeneration within the ON.

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Disclosure statement

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