CUBIC-f: An optimized clearing method for cell tracing and evaluation of neurite density in the salamander brain

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A B S T R A C T

Background: Although tissue clearing and subsequent whole-brain imaging is now possible, standard protocols need to be adjusted to the innate properties of each specific tissue for optimal results. This work modifies exiting protocols to clear fragile brain samples and documents a downstream pipeline for image processing and data analysis.

New Method: We developed a clearing protocol, CUBIC-f, which we optimized for fragile samples, such as the salamander brain. We modified hydrophilic and aqueous’ tissue-clearing methods based on Advanced CUBIC by incorporating Omnipaque 350 for refractive index matching.

Results: By combining CUBIC-f, light sheet microscopy and bioinformatic pipelines, we quantified neuronal cell density, traced genetically marked fluorescent cells over long distance, and performed high resolution characterization of neural progenitor cells in the salamander brain. We also found that CUBIC-f is suitable for conserving tissue integrity in embryonic mouse brains.

Comparison with exiting methods: CUBIC-f shortens clearing and staining times, and requires less reagent use than Advanced CUBIC and Advanced CLARITY.

Conclusion: CUBIC-f is suitable for conserving tissue integrity in embryonic mouse brains, larval and adult salamander brains which display considerable deformation using traditional CUBIC and CLARITY protocols.

1. Introduction

Through an array of tissue clearing methods it is now possible to image large biological samples, including regenerating axons (Ertürk et al., 2012; Hilton et al., 2019; Lindsey et al., 2018) and whole embryos (Belle et al., 2014; Gómez-Gavirio et al., 2017; Masselink et al., 2019). Tissue clearing is reliant on matching the refractive index of the sample to the medium it is imaged in. The refractive index of a tissue can be described by sample volume and the polymer gel properties of the tissue. Importantly, tissues do not need any exogenous polymer to behave as a polymer gel (Murakami et al., 2018; Ueda et al., 2020). Thus, the transparency is reached by altering refractive index by changing polymer gel properties or expansion of the tissue volume. Additional processes such as lipid removal, decalcification and protein conformation changing reagents can all affect the refractive index of the tissue (Kim et al., 2018; Susaki et al., 2014; Tainaka et al., 2018; Tomer et al., 2014).

But as each tissue within each species has specific extracellular matrix composition, different polymer gel properties optimisation is required when applying tissue clearing protocols to new tissue samples. Tissue clearing when coupled with light sheet microscopy has allowed for unprecedented speed of acquisition of large tissue volumes, while still achieving good resolution, comparable to confocal microscopy. Thus this combination is ideal for neurite tracing, neurite density and big sample volumetric studies (Godefroy et al., 2020; Kathuriru and Lichtman, 2007; Liu et al., 2018; Susaki et al., 2015; Tomer et al., 2014).

Salamanders are capable of regenerating several body parts including neural structures (Amamoto et al., 2016; Berg et al., 2011; Joven et al., 2018; Maden et al., 2013; Parish et al., 2007). However, methods to both qualitatively and quantitatively assess neuronal regeneration in terms of neurogenesis and innervation have been

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limited. In the present study, we have optimised the tissue clearing method Advanced CUBIC for the salamander brain and compared its performance with Advanced CLARITY. Using this optimized protocol that we denoted CUBIC-f, we traced genetically labelled neuronal fibres at single cell resolution, quantified the degree of neuronal loss upon chemical ablation of dopamine neurons, and gained new insights into the morphology of neural progenitor cells that line the ventricular system of the salamander brain. In addition, we found that CUBIC-f is superior for preserving embryonic mouse brain tissue.

2. Experimental procedures

2.1. Animal models

Experiments were performed according to guidelines from the European Community and the local ethics committee. *Pleurodeles waltl* were used in this study, raised in our colony, as described in Joven et al. (2015). *Pleurodeles* transgenic salamanders used were described in Joven et al. (2018). Adult mice were wildtype, age ~60 days, and from a mixed genetic background C3H/C57BL6. Embryonic mice were wildtype, embryonic stage E15.5, and from SWISS strain (Janvier Labs, BjOrl:SWISS).

2.2. Dopaminergic chemical ablation

We performed dopaminergic lesions by intraventricular injection of 6-OHDA as described previously (Kirkham and Joven, 2015). 1.5 μl of 0.6 μg/μl solution of 6-OHDA with 1:25 diluted Fluorescent sodium salt (Sigma, F6377) was injected per Pleurodeles stages 51–53. Sham animals were injected with corresponding volumes of 0.9 % NaCl. Animals were sacrificed at 7 days after lesioning.

2.3. Electroporation

The plasmids CMV-EGFP-C1 and CMV-ptdTomato-N1 (Takara Bio USA), were purified using zymoPURE plasmid maxiprep kit (Zymo Research, D42025). Plasmid DNA was injected intraventricularly at a concentration of 2 μg/μl in 1X PBS, as described previously (Joven et al., 2018). The ventral or lateral part of the ventricles electroporated (Nepagene) using two alternative settings or the pore forming pulse. High-voltage: pore forming pulse of 135 mV/cm for 50 ms. Low-voltage: pore forming pulse to 100 mV/cm on 55 ms off. Followed by the 5 transfer pulses of 40 mV/cm for 50 ms with a gap of 999 ms and a decay of 10 % between the pulses. Animals were sacrificed at 3-, 14- and 28-days post electroporation.

2.4. Tissue processing

Salamanders were anesthetized by placing them in a solution in 0.02 % MS222 (Sigma). Heads were immersed in 4 % formaldehyde solution (Millipore, 100496) for 12–24 h at 4 °C, followed by washing with PBS/0.01 % (wt/vol) sodium azide. Adult mice were sacrificed by CO₂ inhalation, followed by cervical dislocation. The whole head or embryo was taken and placed in 4 % formaldehyde solution (Millipore, 100496). All brains were then dissected, including careful removal of the meninges, and placed back in 4 % formaldehyde solution for 1–4 h, followed by washing with PBS/0.01 % sodium azide (wt/vol). The samples were then tissue cleared or stored for later use by placement in sucrose 30 % (wt/vol) for 24 h and then frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek) at −80 °C. After thawing, the samples were washed in PBS at 37 °C prior to use.

2.5. Clearing protocols

Advanced CLARITY was performed as described in Tomer et al. (2014). The following specific conditions were used: i) hydrogel monomer (HM) solution contained 6 % (wt/vol) acrylamide final concentration; ii) Incubation in SDS/borate clearing (SBC) buffer was of 9 days at 45 °C; iii) Primary and secondary immunostaining for 2 days. Advanced CUBIC was performed as described in Susaki et al. (2015). A simple immersion clearing method was also evaluated as this has been shown to clear small neuronal samples efficiently (Loren et al., 2019). Samples were placed iohexol solution, Omnipaque 350 (Refractive index = 1.45761, GE Healthcare Inc) at 37 °C while being rotated (HulaMixer, ThermoFisher) at 30 rpm for 4 days.

2.6. CUBIC-f

CUBIC-f is based on the protocol and reagents from Susaki et al. (2015), with the following changes: i) Decrease of the volume of reagents used per sample; ii) Shortening of all incubation times; iii) Removal of intermediary steps and reagent 2 step. Reagent 1 and reagent 2 are described in Susaki et al. (2015). For the sake of clarity and reproducibility we describe the whole CUBIC-f protocol below.

Plastic teaspooos (Office Depot, Sweden, item number: 0552922) were used to handle the samples on every step of the protocol to prevent morphological damage. Each salamander brain or embryonic mouse brain was incubated in 1 ml of degassed reagent 1 (25 wt % urea (Sigma), 25 wt% Quadrol (N,N,N’,N’-tetra-kis(2-hydroxypropyl) ethylenediamine) (Sigma), 15 wt% Triton X-100 (Nacalai Tesque, Japan)) at 37 °C while being agitated using a rotator (HulaMixer, ThermoFisher) on 30 rpm for a total of 4 days. Reagent 1 was renewed at 2 days. Fourteen ml of reagent 1 were used for adult mouse brains. For antibody labelling, brains were washed in PBS/0.01 % sodium azide (wt/vol) 3 times for 2 h. Follows by each sample being incubated at 37 °C for 2 days in 750 μl of primary antibody in CUBIC block solution 1 (0.1 % (v/v) Triton X-100 (Nacalai Tesque, Japan), 0.5 % (w/v) bovine serum albumin (Sigma) 0.01 % (w/v) sodium azide (Sigma)) while being agitated using a rotator (HulaMixer, ThermoFisher) at 30 rpm. The samples were washed 5 times 30 min each in PBS/0.1 % Triton X-100 (vol/vol) at 37 °C. Next samples were incubated in 750 μl of the corresponding secondary antibody in CUBIC block solution 2 (0.1 % (v/v) Triton X-100 (Nacalai Tesque, Japan), 0.1 % (w/v) bovine serum albumin (Sigma), 0.01 % (w/v) sodium azide (Sigma)) while being agitated using a rotator (HulaMixer, ThermoFisher) on 30 rpm for 2 days at 37 °C. Samples were washed 5 times for 30 min in PBS/0.1 % Triton X-100 (vol/vol) followed by sample degassing.

2.7. Antibodies

For wild type and 6-OHDA-ablated samples analysed by light sheet microscopy for both CUBIC and CLARITY clearing methods, the primary antibodies were rabbit anti-Tyrosine Hydroxylase (TH; Millipore, AB152) 1:500 or mouse anti-Glia fibrillary acidic protein (GFAP) conjugated with cy3 (Sigma, C9205) 1:500. Secondary antibodies were donkey anti-rabbit Alexa Fluor 488 (Jackson ImmunoResearch, 711-546-152) 1:500 or donkey anti-rabbit cy3 (Jackson ImmunoResearch, 711-166-152) 1:500.

Electroporated samples analysed by light sheet microscopy were labelled with either goat anti-tfTomato (Sicgen, AB8161) 1:200 or goat anti-GFP (Abcam, ab6673), followed by the secondary antibodies donkey anti-goat cy3 (Jackson ImmunoResearch, 705-166-147) or donkey anti-goat Alexa Fluor 488 (Jackson ImmunoResearch, 705-546-147). Electroporated samples analysed by confocal microscopy were labelled with mouse anti-TH and donkey anti-mouse DyLight 405 (Jackson ImmunoResearch, 715-476-150).

2.8. Refractive index matching

In all tissue clearing methods performed in this study we used the SeerDB2 refractive index matching solution, iohexol solution Omnipaque 350 (Refractive index = 1.45761, GE Healthcare Inc), instead of the
solutions reported in the original Advanced CUBIC (Reagent 2) and Advanced CLARITY (FocusClear) protocols (Ke et al., 2016; Susaki et al., 2015; Tomer et al., 2014). This refractive index matching solution was renewed 3 times at room temperature before imaging for a period of 1.5 days for custom Advanced CLARITY and of 6–10 h for the CUBIC-based protocols.

2.9. Mounting

We found the classical mounting methods on the Zeiss Z.1 microscope to be suboptimal for salamander brain samples, given their small size and elongated shape. Methods using sample embedding require preparing the embedding gel in the same refractive index matching solution that the sample is immersed in. This is technical challenge due to the viscosity of these solutions and to the required degree of accuracy needed to achieve the final refractive index (Flood et al., 2013). Moreover, these solutions need to be placed on the sample while warm and before they polymerize, which can lead to sample damage due to high temperature. Another classical method for sample mounting is placing the sample on a hook but given small dimension of the larvae brains this could easily disturb the light sheet path. The use of simax tubes instead of the side chamber door classically used for access to the sample mounting procedure. The pictures from the mounting procedure were taken with an iPhone 5 (Apple).

2.10. Microscopy

Pictures of the different stages of the tissue clearing procedure were taken on a M80 stereomicroscope (Leica) equipped with a IC80 HD camera (Leica) and software Leica acquire v3.4.1.

A light sheet Z.1 microscope (Zeiss) with ZEN 2014 software (Zeiss) was used for imaging of the cleared brain samples. The imaging chamber was filled with the refractive index matching solution Omnipaque 350 (GE Healthcare Inc) and this was renewed daily. All acquisitions were performed with a light sheet Z.1 illumination optics 10x/0.2NA, detection optics 20x/1.0NA (clearing n = 1.45, Zeiss) with the refractive index correction collar set to 1.457, zoom set up to 0.71, frame size of 1920 × 1920 (618.3 × 618.3 μm) pixels and 16-bit depth. In the cases where tile scanning was required the camera field of view was cropped by 10 %, yielding a 1728 × 1728 (556.4 × 556.4 μm) pixel frame size. For tile region acquisitions we set a volume of interest (VOI) spanning the average intensity of each stack slice and n is the total pixel count for each stack slice, as previously described in Yu et al. (2017). The calculation formula directly normalizes to the background regions such as the interior of the ventricle and the regions outside of the brain, by taking the average of each slice in consideration. This script removes the zero intensity values introduced by dataset rotation in Fiji, before performing the contrast calculation. Bioinformatic pipelines are available upon request.

2.11. Long term sample storage

To store CUBIC samples for later reimaging we washed and placed them in PBS/0.01 % sodium azide (wt/vol). For Advanced CLARITY samples, we washed and stored them in PBS/0.1 % Triton X-100 (vol/vol) /0.01 % sodium azide (wt/vol). All samples were kept at 4 °C and protected from light. For reimagining of these samples, we placed them back into Omnipaque 350, as described in the refractive index matching subheading prior to image acquisition.

2.12. Image stitching, file conversion and processing

CZI format files were imported into Arivis 2.12.1 (Arivis AG). Tiles were aligned manually from Maximum Intensity Projections (MIPs) of variable thickness, followed by stitching and export to TIFF. The TIFF series was then converted to IFS format with the Imaris file converter 8 and appropriate input of the correspondent X, Y and Z voxel sizes to prevent 3D deformed visualization. IMS files were visualized in Imaris 8. MIPs and spherical volumes were built with the Imaris orthogonal and spots tool, respectively. Neurite tracing was performed with the Imaris filament tracer tool, with the auto path and torch options. Imaris was used to quantify dendrite length, sholl interaction, and branch depth. The degree of branching was evaluated using the imaris tool+ Filament - Full Branch Depth (sum), which evaluates the number of branch points or bifurcations within a traced structure. IMS files were exported into TIFF and downsized to 10 % its size for contrast and intensity measurements. TeraStitcher was used as described previously (Bria and Iannello, 2012).

2.13. Bioinformatics

To facilitate the selection of VOIs we rotated the 10 % downsized TIFF series in Fiji version 1.0 (ImageJ version 1.51n) so that the TH+ rostral commissure would be placed horizontal in all datasets. This rotation introduces black regions in the edges of the pictures, whose intensity values are exact zeros. Since these values are still lower than the actual image background in the original images, we remove the introduced black regions by filtering out for zero intensity values in our subsequent macros.

We wrote customized ImageJ macros to measure the intensity of every pixel for every stack slice of our datasets within defined VOIs made from a rectangular horizontal selection; the Z cropping was defined at the last stack slice showing the rod where the brain was mounted and the first stack slice showing the desired VOI. These measurements were exported to CSV files. This macro was run in Fiji version 1.0 with ImageJ version 1.51n and the 10 % downsized versions of our datasets were used.

We then wrote a R (RStudio 1.0.136 with R version 3.3.2) script to calculate the normalized contrast of each slice of our VOIs according to the formula

\[
\text{Normalized contrast} = \sqrt{\frac{\sum (I - I_{\text{mean}})^2}{n - 1}}
\]

where I represents the grayscale value for each pixel, I_{\text{mean}} represents the average intensity of each stack slice and n is the total pixel count for each stack slice, as previously described in Yu et al. (2017). The calculation formula directly normalizes to the background regions such as the interior of the ventricle and the regions outside of the brain, by taking the average of each slice in consideration. This script removes the zero intensity values introduced by dataset rotation in Fiji, before performing the contrast calculation. Bioinformatic pipelines are available upon request.
Fig. 1. Morphological comparison of salamander and mice brains after various clearing methods. (A) Representative images illustrating the morphology of brain samples after Advanced CUBIC protocol. Embryonic mouse and salamander samples become distorted, while adult mouse brain samples retain their morphology. (B) Representative images illustrating the morphology of brain samples after CUBIC-f protocol. All samples cleared with CUBIC-f retain their morphology, while adult mice brains become insufficiently cleared. Each grid square = 775 μm. The dashed yellow line indicates the contours of highly transparent samples for easier visualization.
2.14. Statistical analyses

The statistical tests: Welch, Two Sample t-test and one-way ANOVA with Tukey’s post-hoc analysis were performed in RStudio 1.0.136 with R version 3.3.2. Graphs were prepared in Prism 7.0a (GraphPad).

3. Results

3.1. CUBIC-f minimizes tissue clearing times and brain deformation

In order to study neurites in whole salamander brain volumes, we evaluated the performance of two pre-eminent tissue clearing techniques in the field, Advanced CUBIC and Advanced CLARITY on larval (stage 51–53) salamander brains (Susaki et al., 2015; Tomer et al., 2014). Advanced CUBIC produced a high degree of transparency, but severely compromise tissue integrity (Fig. 1A) to an extent that the sample was unmanageable. We asked whether this deformation was due to the amphibian or embryonic nature of the samples, since the Advanced CUBIC protocol was originally developed for adult mice brains. While adult mice brains cleared in Advanced CUBIC retained their tissue integrity and cleared well (n = 4), the embryonic mouse brains (3 out of 4) and all larval samples (n = 4) processed became deformed (Fig. 1A). Adult salamander brains also underwent considerable deformation when cleared in Advanced CUBIC (Fig. 1A) (2 out of 4). We concluded that Advanced CUBIC is not an ideal method for the clearing of dissected embryonic mouse brains and larval as well as adult salamander brain. These observations suggest that the adult salamander brains might share characteristics in tissue composition with mouse embryonic brain, which could influence the integrity of the sample during tissue clearing.

Hydrophilic or aqueous’ tissue-clearing methods such as CUBIC have a two-step process, delipidation followed by refractive index matching. These steps can be complemented with decolourisation and or staining protocol (Ueda et al., 2020). Reagent 2 in the Advanced CUBIC protocol (Susaki et al., 2015) is required for refractive index matching, however salamander brains’ deformation and tissue damage mainly occurred during incubation in reagent 2 (Fig. 1A). We investigated whether this could be substituted for refractive index matching solution Omnipaque 350, a ready-to-use iohexol based solution (Ke et al., 2016; Yang et al., 2014).

We estimated the adult salamander brain to be volumetrically ~46 times smaller than an adult mouse brain and reduced the amounts of
reagents used by a factor of 10. Lastly, we shortened all the clearing and staining times to reduce sample fragility, and to shorten the duration of the protocol. From start to imaging of samples labelled with secondary antibody, we shortened the CUBIC protocol from 16 days to 9 days. These modifications are similar to the modifications made in Gómez-Gaviro et al. (2017) but different in terms of replacing reagent 2 with Omnipaque 350 (see details in Methods section). We named this version of Advanced CUBIC, initially optimized for salamander brains, CUBIC-fragile (CUBIC-f). Further protocol details are outlined in the Experimental Procedures section. We observed that CUBIC-f retains sample integrity of all embryonic/larval and adult samples, both of mice and salamanders (n = 4 for all groups) (Fig. 1B & 2A). However, and as expected, adult mouse brains were insufficiently cleared due to the shortened exposure to clearing reagents (Fig. 1B). This could be due to both sample size and composition as when 3–5 mm sections of adult mouse brain where cleared with CUBIC-f large regional variation in tissue transparency within the same section could be observed (Supp. Fig. 1A). These observations suggest that tissue composition could be equally important as the size or thickness of the tissue in determining tissue clearing protocols.

We wanted to see if further simplification of the protocol could give similar results. Immersion in iohexol solutions similar to Omnipaque 350 had been successfully used to clear thick brain sections (Loren et al., 2019). Interestingly, immersion in Omnipaque 350 for extended periods of time cleared larval salamander brains without any sample deformation (n = 5), but not adult salamander brains (n = 4) (Supp. Fig. 1B). However, when larval brains from GFPloxP-Cherry transgenic salamanders cleared in Omnipaque 350 were imaged, image quality was drastically reduced with increased tissue depth. In comparison, samples cleared in CUBIC-f had reduced GFP signal but better image quality in terms of resolution (Supp. Fig. 1C–F) (n = 4).

3.2. Advanced CLARITY perform sub optimally for whole volume imaging of salamander brains compared to CUBIC-f

Advanced CLARITY is a hydrogel-based method for tissue clearing. To assess CUBIC-f further we compared salamander brains cleared with Advanced CLARITY to salamander brains cleared with CUBIC-f. Salamander brains were cleared with Advanced CLARITY (Tomer et al., 2014) with one modification: 6 % (wt/vol) acrylamide in the hydrogel monomer solution was used to reduce tissue curling in the relative small sample size of the salamander brain. For refractive index matching we used Omnipaque 350. This protocol successfully rendered the samples transparent by the end of the refractive index matching step (Fig. 2A). However, salamander samples were sensitive to deformation and breakage during the SBC buffer step, but 6 out of 14 brains could still be stained, mounted and imaged. The reason for this sensitivity is unclear. For appreciation of antibody depth penetration and intensity of staining signal, ependymoglial cells (neural stem cells lining the ventricular system in the salamander brain (Kirkham et al., 2014; Parish et al., 2007), were labelled with an antibody against the glial fibrillary acidic protein (GFAP). GFAP labelling was homogeneous across depth and different regions of the brain (Supp. Fig. 2A).

Due to challenges in tissue size and integrity we developed a novel mounting method, which is ideal for imaging of small samples in light sheet Z.1 microscopes. Samples were mounted on a thin metal rod and inserted into the Z.1 microscope through the upper opening instead of the conventional side door (Fig. 2B and C). This mounting method provides high flexibility in imaging small tissues from the desired viewing angle and minimizes disturbance to the light sheet and difficulty of mounting. We further demonstrate this mounting procedure to be applicable for the mounting of multiple brain samples on the same rod (Supp. Fig. 2B). When using Multiview acquisition tools, we were able to screen and image multiple brains with one single mounting and setup of imaging settings.
Cleared samples were imaged from ventral to dorsal through the midbrain, and GFAP features could be distinguished at all depths in samples cleared with both Advanced CLARITY and CUBIC-f, (Fig. 2D & E, Supp. Figs. 2C–E). However, it was apparent that the signal intensity was higher and the resolution better at the deepest regions in samples cleared with CUBIC-f compared to Advanced CLARITY. This loss of signal intensity could be due to a decrease in antibody penetration. Even without the use of antibodies as a fluorescent source, GFP signal was lower in the samples cleared with Advanced CLARITY when quantified in GFP-loxP-Cherry transgenic salamanders (Supp. Fig. 2F–G). This data indicates that the signal reduction in the Advanced CLARITY cleared samples was due to reduced light penetration at increasing tissue depths.

In order to quantify the fluorescent signal in light sheet acquired data, we performed normalized contrast and intensity measurements (Yu et al., 2017). Consistent with our qualitative observations, CUBIC-f quantification showed higher normalized GFAP contrast values in all samples compared to samples prepared with Advanced CLARITY (Fig. 2F and G). Indeed, CUBIC-f exhibited over 5 times higher normalized contrast value compared to Advanced CLARITY (Fig. 2G). Since the contrast method takes the whole of each stack slice into account and the GFAP projection pattern slightly varies across the stack, we chose to confirm our result by the measurement of local intensity. We achieved this by measuring the intensity of six spherical volumes of 100 μm diameter positioned close to the ventricle at different depths (Supp. Fig. 2E). In line with our previous finding with the contrast method, we found that the GFAP signal intensity decreased with depth and was higher in CUBIC-f compared to Advanced CLARITY cleared samples (Supp. Fig. 2E).

We next examined neurites of dopamine neurons in the salamander midbrain using CUBIC-f and Advanced CLARITY. TH⁺ cell bodies and neurites in the ventral midbrain of Pleurodeles brains were more visible in CUBIC-f cleared brains compared to Advanced CLARITY cleared brains (Fig. 3A–C, Supp. Video 1). Furthermore, CUBIC-f samples also had a higher average fluorescence intensity in the ventral midbrain after labelling with an antibody against TH (average fluorescence intensity CUBIC-f: 3090 SEM ± 800, Advanced CLARITY: 910 SEM ± 190). TH⁺ neurons occasionally appear beaded; this appearance is likely due to the antibody labelling as there is no correlation with sample preparation or treatment. For example, TH neurons labelled in 20 μM cyro-sections can have similar appearance (Supp. Fig. 3H). In conclusion, CUBIC-f is a superior method for clearing and imaging of salamander brains as it minimizes protocol time and brain deformation and increases staining quality across depth (Fig. 3).

3.3. Quantification of dopaminergic lesions in whole volumes of CUBIC-f cleared salamander brains using contrast and intensity measurements

We next aimed to quantify the degree of neuronal lesion after ablation of dopamine neurons using the selective toxin, 6-OHDA (Parish et al., 2007) in tile scanned salamander brains. Tile scanning requires later stitching of the overlapping areas between tiles, which can create artefacts between tiles. In order to minimize these artefacts, we tested two stitching software, Arivis and Tera Stitcher (Bria and Iannello, 2012). For Arivis we performed manual tile alignment while for Tera...
Stitcher we used different combinations of blending and SPIM correction options. Arivis produced the most optimal result in our samples, by producing the least signal intensity and alignment artefacts between tiles, and was therefore our choice throughout the rest of our study (Supp. Fig. 3G). The scanned region was delimited from the most caudal dopaminergic cell in the ventral midbrain to the most caudal dopaminergic cell in the olfactory bulb of the forebrain. The nucleus accumbens neuropil was used as an extra positional indication (Supp. Fig. 3, Supp. Video 2). The final stitched datasets in the ventral midbrain-striatum region of brains amounted to 91–221 gb of data and took 35–85 min of imaging time per brain. The Y axis distance between the most caudal cell of the ventral midbrain and the most rostral part of the striatum was about 2402 μm, while the whole scanned volume was about 2650 μm in the same axis.

We subsequently measured normalized contrast within these VOIs as already described. Before quantification, Z slices where the metal rod from the sample mounting was apparent and Z slices at the top of the stack, where mostly background was present, were removed. In 6-OHDA lesioned brains, 7 days post ablation, we detected decreased TH+ neurite and cell body density in both the midbrain and forebrain regions (Fig. 4A & B, Supp. Video 3 & 4). By doing contrast quantifications we estimated dopaminergic density to be about 3.3-fold lower in the forebrain, and 1.9-fold lower in the midbrain region of these lesioned brains (Fig. 4A & B). We concluded that CUBIC-f, coupled with light sheet microscopy and bioinformatics pipelines, is an easy-to-apply method for quantification of the dopaminergic ablation in salamander brains.

Fig. 5. Electroporation and tracing of ependymoglial cells and their progeny in the salamander brain after clearing with CUBIC-f. (A) Ependymoglial cell in the ventricular layer of the ventral midbrain, 14 d after electroporation (low-voltage) with a CMV-GFP plasmid. (B) Six ependymoglial cells in the ventricular layer of the forebrain, 14 d after electroporation (high-voltage) with CMV-tdTomato, and a corresponding tracing diagram of their radial projections. (C) Branching diagram of each ependymoglial cell traced in B, pseudo-coloured by the degree of branching. (D) Total length of all dendrites for each ependymoglial cell traced in B, with their respective colours. (E) Distance from cell body to each radial projection terminal, across branch depth, for each ependymoglial cell traced in B. (F) Number of Sholl intersections at increasing sphere radius for the neurites branching from each ependymoglial cell traced in B. (G) Tracing of a neuron derived from ependymoglial cell, 28 d after electroporation, from the cell body in the mantle zone of the forebrain (white arrowhead, —nucleus accumbens) to its last projection terminal in the midbrain (—tegmentum). (H) MIPs and tracing representations of the cell body (red arrowhead) and longest terminal for the cell represented in G, in red. Note the additional labelled cells (yellow and green arrowheads) in the longest terminal projections. (I) Distance plotted from the cell body to each radial projection terminal, across branch depth, for the bipolar cell traced in G. The longest traced terminal has the length of 3647 μm. (J) GFP+ ependymoglial cell (arrow-head) and ‘TH+’/GFP+ cell (arrow) with neurites, 14 days after labelling with CMV-GFP electroporation. The same cells are represented in Supp. Fig 4C. Scale bars = 100.

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3.4. Light sheet imaging of brains cleared with CUBIC-f allows tracing of long-range projections, tracing of neurons, and characterization of ependymoglial cells in the salamander brain

We next asked whether CUBIC-f, in combination with light sheet microscopy, would facilitate tracing and characterization of neurons and their progenitors. To label cells, we injected CMV-EGFP-C1 or CMV-ptdTomato-N1 constructs intraventricularly, followed by electroporation. We observed that, in line with previous studies, this protocol marked exclusively ependymoglial cells in the ventricular layer at 3 days post electroporation (Supp. Fig. 4A & B, Supp. Video 5) (Joven et al., 2018). Individual ependymoglial projections could be visually mapped to a specific cell body (Fig. 5A, Supp. Video 6). We traced the radial extensions of 6 ependymoglial cells from their cell bodies at the ventricular layer to each of their terminals projecting towards the pial surface (Fig. 5B,C, Supp. Video 7). By doing so we successfully extracted parameters that highlight previously unappreciated heterogeneity among ependymoglial cells. We found that different ependymoglial cells had different degrees of branching, as shown by the filament branch depth parameter (Fig. 5C). Likewise, the projections’ total length varied greatly from cell to cell, as did the distribution of distances from their origin to each terminal (Fig. 5D-E). The Sholl intersections method allows for measuring cell protrusion density across the increasing radius of a sphere (Langhammer et al., 2016; Sholl, 1956). Using this method, we observed that some ependymoglial cells show an increase in extension branching at lower sphere radius (green cell), while others start branching later (red and turquoise cell, Fig. 5F).

Next we labelled ependymoglial cells during larval neurogenesis (stage 51–53 (Joven et al., 2018). 14 days post electroporation of CMV-EGFP-C1 and CMV-ptdTomato-N1 constructs, we could indeed find GFP+ and tdTomato− cells outside of the ventricular layer, indicating the electroporated ependymoglial cells or their progeny migrated away from the ventricle (Fig. 5J Supp. Video 9).

We encountered GFP+ positive cells away from the ventricular layer also at 28 days post electroporation. We traced a bipolar cell from its cell body to all of its terminals (Fig. 5G & H, Supp. Video 8). The maximum traced distance was a length of 3647 μm from its cell body next to the nucleus accumbens, close to the striatum, to the end of its projection in the tegmentum, close to the ventral midbrain dopaminergic cell group (Fig. 5I). This demonstrates that CUBIC-f, coupled with light sheet microscopy, allows for the tracing of long-distance neurites.

4. Discussion

The field of tissue clearing has experienced tremendous advances after the development of Scale in 2011 and CLARITY in 2013 (Chung et al., 2013; Hama et al., 2011). Furthermore, advances in light sheet microscopy have facilitated the imaging of large transparent samples with little sacrifices in resolution. CUBIC-f amalgamates these developments and makes it possible to image cleared fragile brains at high resolution (Figs. 1 and 3). Moreover, it does so in shorter times and lower reagent amount than the method we used as a model for optimization, Advanced CUBIC (Susaki et al., 2015). CUBIC-f also allows for the quantification of neuronal lesions in salamanders and neurite tracing to distances over 3600 μm (Fig. 4). In the future we envisage that it should be possible to combine this imaging platform with transgenic salamanders, in which specific cell populations can be stably and conditionally labelled (Joven et al., 2019) and to reveal patterns of reinnervations.

Imaging of the neural progenitors, the ependymoglial cells, revealed subpopulations based on the properties of projections, such as branch level, length, targets and Sholl intersections. It will be interesting to extend previous studies with this methodology and correlate the findings to gene expression patterns that previously identified distinct ependymoglial cell populations in the salamander brain (Kirkham et al., 2014). In addition, developmental studies, such as those we performed previously (Joven et al., 2018) will shed light on how maturation of ependymoglial cells correlates with morphological changes. This is especially relevant for the fact that radial projections have been found to promote neuronal migration and recovery as a response to brain injury also in the mouse neonatal cortex (Jinnou et al., 2018). The projections of ependymoglial cells could therefore provide a location scaffold for their progeny to reach their targets. Characterization of different types of ependymoglial cells could therefore help with the mechanistic understanding of these processes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.jneumeth.2020.109002.

References

Aramoero, R., Huerta, V.G.L., Takahashi, E., Dai, G., Grant, A.K., Fu, Z., Arlotta, P., 2016. Adult axolotls can regenerate original neuronal diversity in response to brain injury. Elife 5. https://doi.org/10.7554/eLife.1996.
Belle, M., Godfrey, D., Dominici, C., Heitz-Marchaland, C., Zelina, P., Hellal, F., Bradke, F., Chedotal, A., 2014. A simple method for 3D analysis of immunolabeled axonal tracts in a transparent nervous system. Cell Rep. 9, 1191–1201. https://doi.org/10.1016/j.celrep.2014.10.037.
Berg, D.A., Kirkham, M., Wang, H., Fritsén, J., Simon, A., 2011. Dopamine controls neurogenesis in the adult salamander midbrain in homeostasis and during regeneration of dopaminergic neurons. Cell Stem Cell 8, 426–433. https://doi.org/10.1016/j.stem.2011.02.003.
Bria, A., Iannello, G., 2012. TeraStitcher - a tool for fast automatic 3D-stitching of terawoxel-sized microscopy images. BMC Bioinf. 13 https://doi.org/10.1186/1471-2105-13-316.
Chung, K., Wallace, J., Kim, S.Y.Y., Kalpandasundaram, S., Andalman, A.S., Davidson, T., J. Mirzabekov, J.J., Deisseroth, K., Zalocusky, K.A., Mattis, J., Denisin, A.K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., Deisseroth, K., Zalocusky, K.A., Mattis, J., Denisin, A.K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., Deisseroth, K., 2013. Structural and molecular interrogation of intact biological systems. Nature 497, 332–337. https://doi.org/10.1038/nature12107.
Erkirk, A., Mauch, C.P., Hellal, F., Förster, F., Keck, T., Becker, K., Jahrhing, N., Steffen, H., Richter, M., Hübené, M., Kramer, E., Kirchhoff, F., Dölt, H.U., Bradke, F., 2012. Three-dimensional imaging of the unsectioned adult spinal cord to assess axon regeneration and glial responses after injury. Nat. Med. 18, 166–171. https://doi.org/10.1038/nm.2600.
Flood, P.M., Kelty, R., L., G.H., E.G., R., 2013. ZEISS Lightsheet Z.1 Sample Preparation. Zeiss White Pap. September, 1–34.
Godfrey, D., Boukhzar, L., Dubessy, C., Montero-Hadjadjie, M., Yon, L., Eiden, L.E., Anatou, Y., 2020. Three-dimensional mapping of tyrosine hydroxylase in the transparent brain and adrenal of prenatal and pre-weaning mice: comprehensive
