Salamander-Eci: An optical clearing protocol for the three-dimensional exploration of regeneration

Cristina Subiran Adrados1 | Qinghao Yu1 | Lizbeth Airais Bolaños Castro1 | Luis Alberto Rodriguez Cabrera1,2 | Maximina Hee Yun1,3

1CRTD/Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany
2Department of Pediatrics, Neonatology and Pediatric Critical Care Medicine, Technische Universität Dresden, University Hospital and Medical Faculty Carl Gustav Carus, Dresden, Germany
3Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany

Correspondence
Maximina Hee Yun, CRTD/Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany.
Email: maximina.yun@tu-dresden.de

Funding information
European Regional Development Fund (EFRE); Center for Regenerative Therapies Dresden, Grant/Award Numbers: DFG EXC 168, DFG FZ 111

Abstract

Background: Salamander limb regeneration is a complex biological process that entails the orchestration of multiple cellular and molecular mechanisms in a three-dimensional space. Hence, a comprehensive understanding of this process requires whole-structure level explorations. Recent advances in imaging and optical clearing methods have transformed the study of regenerative phenomena, allowing the three-dimensional visualization of structures and entire organisms.

Results: Here we introduce Salamander-Eci, a rapid and robust optical clearing protocol optimized for the widely used axolotl model, which allows simultaneous immunohistochemistry and Click-chemistry detection with minimal volume disruption. We provide examples of its application, from whole larva to adult limbs and organs, and complement it with an image analysis pipeline for volumetric cell quantification. Further, we offer a detailed 3D quantitation of cell proliferation throughout axolotl limb regeneration.

Conclusions: Salamander-Eci enables the comprehensive volumetric analysis of regenerative phenomena at both local and systemic levels.

KEYWORDS
axolotl, click-chemistry, development, optical clearing, regeneration, salamander

1 | INTRODUCTION

The study of developmental, physiological and pathological mechanisms in biological specimens has traditionally relied on two-dimensional histological analyses, in which samples are cut into thin sections that are subsequently processed to examine the inner workings of biological tissues and organs. The process, however, is labor-intensive and time consuming, and precise information pertaining to the morphology and position of individual cells within the whole organism is often lost. Moreover, the application of histology to studies of large and complex systems requires sectioning followed by reconstruction of three-dimensional structures from two-dimensional information.1,2 This process is intrinsically limited by tissue processing, in which imperfect sectioning leads to loss of information during reconstruction.3
Tissue clearing methods have circumvented the need for sectioning and reconstruction, and provide an unbiased way to interrogate complex systems (reviewed in References 4 and 5). Clearing techniques render biological specimens optically transparent while preserving their three-dimensional structure and, when coupled with light-sheet microscopy, offer a powerful method to achieve rapid, volumetric imaging of intact biological samples.\(^8,9\) Furthermore, advances in the past decade led to the ability to combine clearing with a wide array of molecular probing techniques, such as in situ hybridization,\(^8,9,10\) whole-mount antibody stainings,\(^4,5,8-11\) and click-chemistry-based detection. As such, it is now possible to obtain information at sub-cellular resolution within the original tissue context. Thus, clearing approaches are replacing traditional histology methods in the study of complex systems.

Optical clearing techniques achieve transparency of biological samples by reducing the internal differences in refractive index (RI) within the tissue, thereby reducing light scattering. Currently, there are three major approaches to tissue clearing: hydrophobic or solvent-based, such as immunolabeling-enabled three-dimensional imaging of solvent-cleared organs, iDISCO\(^4,5\) and ethyl cinnamate-based (Eci) clearing\(^12\) hydrophilic or aqueous-based, such as Scale\(^13\) and See Deep Brain, SeeDB\(^14\); and hydrogel-based, such as CLARITY and its variants.\(^8,15\) While aqueous-based and hydrogel-based protocols minimize structural loss and better preserve the 3D structure of proteins, solvent-based methods achieve greater levels of transparency, reduce sample processing time and allow permanent preservation of the samples owing to the hardening of the cleared tissues (reviewed in Reference 7).

In the past years, these approaches have been leveraged to achieve optical clearing in a number of species relevant to regenerative biology including the axolotl, *Ambystoma mexicanum*. Second-generation ethyl cinnamate-based clearing (2Eci)\(^11\) and DEEP-Clear\(^10\) have been recently employed to successfully clear axolotl tissues in a manner compatible with immunolabeling as well as with the preservation of endogenous fluorescence derived. However, desirable technical features such as the ability to perform click-chemistry, alone or in combination with other detection methods, as well as pipelines for volumetric cell quantitation, have not yet been reported in the axolotl system.

Here, we present a new clearing method, termed Salamander-Eci, which is a robust pipeline optimized for axolotl tissues. Salamander-Eci enables simultaneous whole-mount immunostaining and click-chemistry detection, with minimal tissue disruption, in a broad repertoire of salamander samples, ranging from whole larva, intact and regenerating limbs, through to large pigmented organs such as spleen.

## RESULTS AND DISCUSSION

### Establishment of Salamander-Eci

In order to develop a fast and effective optical clearing protocol enabling whole-mount antibody staining in axolotl tissues, we combined advantages of both iDISCO and Eci methods, adopting the robust immunostaining procedure from the former and the rapid, non-toxic clearing from the latter. The method developed by Klingberg et al\(^12\) served as the basis for our Eci-based tissue clearing steps, while the traditional iDISCO protocol\(^4\) provided the foundation of the immunolabeling steps. The resulting Salamander-Eci clearing protocol is described in detail in the Experimental Procedures section. Nevertheless, several considerations are noteworthy. Firstly, organic-based clearing protocols substitute the aqueous phase of tissues samples with solvents that match the RI of remaining lipid membranes, by first dehydrating the sample and subsequently immersing in an organic clearing agent. In Salamander-Eci, we used a combination of ethanol as the dehydration agent and ethyl-cinnamate as the organic solvent, to obtain a fast, non-expensive and non-toxic clearing protocol with reduced tissue shrinkage.

Secondly, the timing of every incubation step within the immunolabeling procedure was optimized for staining of axolotl tissue samples. Thirdly, we include the option of combining this clearing approach not only with immunolabeling, but also with click-chemistry and nuclear staining within the same sample, increasing its versatility in the study of biological processes.

Initially, we set out to establish a protocol compatible with whole-mount antibody labeling. Axolotl tissues can exhibit strong autofluorescence in various contexts, which can interfere with the analysis of immunostainings. Therefore, we first determined the level of autofluorescence following Salamander-Eci clearing, in order to identify optimal laser power and excitation wavelengths that maximize signal-to-noise ratio (Figure 1A-P). Of note, ethanol dehydration leads to mild fluorescence-quenching,\(^11\) which contributes to a reduction in background autofluorescence. This analysis indicates that tissue autofluorescence is lowest in far-red wavelengths; thus, fluorophores in this region of the spectrum offer the best signal definition. Nevertheless, note that some structures exhibit high levels of intrinsic autofluorescence in certain channels, such as the pronephros, the embryonic kidney (Figure 1Q-S).

Next, we tested the compatibility of Salamander-Eci with immunostaining of whole axolotl larva and mature limbs, taking advantage of the Car-Act:eGFP and CNP: eGFP transgenic lines.\(^16\) Anti-GFP immunostaining of Car-Act:eGFP larvae resulted in uniform labelling of the larval musculature throughout the organism (Figure 2A,
Movie S1). A similar pattern was observed when staining wild type (wt) larva with an antibody raised against the muscle marker myosin heavy chain (MHC) (Figure 2B). In addition, anti-GFP staining of CNP:eGFP larvae revealed the stereotypical pattern of nerves within the mature axolotl limb (Figure 2C), and allowed us to confirm

**FIGURE 1** Effect of laser power on tissue autofluorescence in optically cleared axolotls following light-sheet imaging. A–P, Snapshot of 3D projection of a cleared axolotl larva in blue channel (A, E, I, M), green channel (B, F, J, N), red channel (C, G, K, O) and far red channel (D, H, L, P) at increasing laser power (10%, 25%, 50%, 75%). Scale bar: 500 μm (n = 3). Q–S, Autofluorescence of the axolotl kidney (white arrowhead in K) in the red spectrum. Snapshot of a 3D rendering of axolotl larva, focused on its pronephros (Ex: 605/50; Em:670/50 nm). Scale bar: 300 μm.
that Salamander-Eci enables combinatorial antibody staining within the same sample, by co-staining with an anti-MHC antibody (Figure 2C). The compatibility of Salamander-Eci with immunolabeling was tested for additional epitopes, as summarized in Table 2.

### 2.2 Preservation of sample size and morphology

Pioneering research introduced 2Eci as a broad-applicability clearing protocol which was also compatible with salamander tissues. Thus, we sought to address the performance of Salamander-Eci in relation to 2Eci. By conducting side-by-side comparisons, we found that Salamander-Eci better preserves the size and morphology of axolotl samples. For this, we analyzed shape preservation and shrinkage in cleared axolotl larvae (Figure 3). Whereas 2Eci led to morphological alterations and sample shrinkage (Figure 3A, H), shrinkage occurred to a lesser extent in samples processed with Salamander-Eci, which also offered better preservation of tissue structure (Figure 3B, I). By measuring the sample area pre and post clearing, we found that the Salamander-Eci protocol

![Table 2](image)

**Table 2**: Summary of compatibility of Salamander-Eci with immunolabeling. Additional epitopes tested include Fetal B-4 (FRC:GFP), Fetal B-4 (FRC:GFP) and B-4 (FRC:GFP)

![Figure 2](image)

**Figure 2**: Salamander-Eci allows 3D visualization of organisms and structures following whole-mount immunostaining. A-C, Representative images of axolotl samples after immunostaining, Salamander-Eci clearing and light-sheet imaging. A, 3D projection of a car-act:eGFP axolotl larva following clearing and subsequent immunostaining with anti-GFP antibody (green) and Sytox-green (grey). B, Overview of a wt axolotl larva following clearing and subsequent immunostaining with anti-MHC antibody (red) and Sytox-green (grey). C, 3D projection of a limb from a cnp:eGFP axolotl following clearing and subsequent immunostaining with anti-GFP antibody (green), anti-MHC (red) and Sytox-green (grey). Scale bar: 500 μm, A, B; and 800 μm, C.
results in 27% average shrinkage while 2Eci results in 41% average shrinkage (Figure 3C). Further, the extent of larval curvature post-processing was significantly lower in Salamander-Eci processed samples than in 2Eci, as evaluated by measuring the deviation angle of the antero-posterior axis (Figure 3D). With regards to tissue clearing
capacity, both protocols result in efficient optical clearing without noticeable differences between them (Figure 3E-G). Thus, Salamander-Eci constitutes a technical advance in terms of sample size and morphology preservation.

### 2.3 Compatibility with click-chemistry

Analysis of cell proliferation is a fundamental tool in the study of biological phenomena such as regeneration.
and development. Thus, we assessed the compatibility of Salamander-Eci with click-chemistry, in particular the detection of 5-ethynyl-2′-deoxyuridine (EdU)-labeled DNA, a common S-phase indicator. To achieve this, we gave axolotl larvae a 1 hour EdU pulse before processing the entire organism using Salamander-Eci. Notably, this revealed active EdU incorporation sites across a broad range of regions in actively growing axolotl larvae, including limbs, brain, eye cups, spinal cord, spleen, and thymus (Figure 4A-C). Staining was consistent and of high penetrance throughout the samples. This is exemplified by 3D reconstructions of the brain, in which several active EdU incorporation sites are observed, including midbrain, hindbrain and optic cups (Movie S2), as well as flow-through analysis of the thymic nodules, which exhibit strong EdU incorporation, in agreement with their lymphopoietic function (Movie S3). Further, analysis of mean EdU and Sytox green intensity vs tissue depth indicates that Salamander-Eci-treated samples do not exhibit noteworthy fluctuations of fluorophore signal along the Z-axis (Figure 4D).

Three-dimensional explorations in the axolotl would benefit from tools for quantitative volumetric analysis. Thus, we developed a semi-automated pipeline for image analysis of cleared samples. In the first step, we performed image pre-processing using a Jython script for the open-source FIJI software to remove the high background signal and to de-noise the images. Imaris software was subsequently used to analyze the image stacks (z-stacks) further. This enabled the volumetric quantitation of EdU⁺ cells relative to sample volume in whole spleen samples (Figure 5A,B, Movie S4) and full limbs (Figure 6, Movie S5, as discussed below). The detailed quantification pipeline is available at https://git.mpi-cbg.de/scicomp.

In addition, we addressed whether Salamander-Eci is compatible with the detection of EdU⁺ cells through click-chemistry together with antibody-mediated antigen detection. This was indeed possible, as shown by the
ability to simultaneously detect EdU⁺ and CD79a⁺ cells (Figure 5C). CD79a is a B lymphocyte marker previously described in the axolotl.¹⁷ The presence of CD79⁺ cells in the axolotl limb is in line with previous studies.¹⁸ Together, these results highlight the versatility of Salamander-Eci, which allows the simultaneous...
2.4 Preservation of fluorescence over time

Sample archiving is not only desirable but also required as part of Good Laboratory Practice guidelines. Thus, we investigated the extent to which fluorescent signals are preserved following Salamander-Eci processing of axolotl tissue samples. Notably, fluorescence derived from either AlexaFluor fluorophores (Figure 6A,C) or Sytox green (Figure 6B,D) remained stable up to 6 months after Salamander-Eci processing. This period of signal preservation is longer than any reported so far. However, it should be noted that, even though no signal decay is detected, the background noise may slightly increase due to the hardening of the samples, a phenomenon that decreases transparency and is common across Eci-based protocols. With regards to sample storage, we recommend to do this in 100% Eci, protected from light, at room temperature and within air-tight containers to prevent oxidation, as advised elsewhere. To sum up, Salamander Eci allows the preservation of fluorophore signals on a long-term basis, facilitating sample archiving and a posteriori analysis.

2.5 Volumetric analysis of cell proliferation during axolotl limb regeneration

To provide an example of the applicability of Salamander-Eci based clearing to the study of regenerative phenomena, we performed a volumetric quantitative analysis of EdU incorporation, an indicator of cell proliferation, through the course of axolotl limb regeneration (Figure 7A-C). For this, we delivered an EdU pulse 1 hour prior to sample collection, processed specimens through Salamander-Eci and quantified the total number of cells in both pre (300 μm proximal to the amputation plane) and post amputation (blastema) regions (Figure 7B). No significant changes in EdU incorporation are observed at 1 and 2 days post amputation, consistent with the notion that these stages are dominated by re-epithelialization, immune recruitment and tissue remodeling processes (Figure 7A,C). As regeneration ensues, significant increases in EdU incorporation are observed, from early bud up to digit formation stages. The proximal-most area to the amputation plane, a source of progenitors for the regenerate, exhibits similar rises in EdU+ cells, albeit to a lesser extent. By the end of regeneration, the extent of EdU incorporation returns to basal levels (Figure 7A,C). Together, this analysis showcases the quantitative possibilities offered by Salamander-Eci and provide a first, three-dimensional insight into EdU incorporation during axolotl limb regeneration.

2.6 Concluding remarks

We hereby provide an optical clearing protocol specifically tailored to axolotl tissues, which can be simultaneously combined with whole-mount immunolabeling and click-chemistry, enabling direct volumetric analysis and quantification. This protocol enables robust antibody diffusion across large samples, whilst circumventing the time-consuming pre-treatment steps required in the original iDISCO method, and replaces toxic solvents with safe and cheap solvents such as Eci, that achieve full transparency rapidly. Of note, it does so while reducing shrinkage and preserving tissue morphology, in comparison with existing alternatives. We envisage that this protocol could be adapted to other salamander models. Importantly, Salamander-Eci is fully compatible with click-chemistry, providing the first example of this application in salamander tissues, thus adding a new tool for in-depth explorations of proliferative phenomena in the axolotl. As such studies would require three-dimensional quantification instruments, we offer a detailed pipeline for volumetric analyses in entire organs and structures, likely applicable to other clearing protocols. To sum up, Salamander-Eci provides a new tool for both qualitative and quantitative three-dimensional explorations of important biological phenomena in the axolotl.

3 EXPERIMENTAL PROCEDURES

3.1 Animal husbandry and procedures

Procedures for care and manipulation of all animals used in this study were performed in compliance with the laws and regulations of the State of Saxony, Germany. Axolotls (A mexicanum) were obtained from the axolotl Facility at TUD-CRTD Center for Regenerative Therapies TU Dresden (Germany) and maintained in individual aquaria at 18°C to 20°C, as previously described. For all animal procedures, axolotls were anesthetized in 0.03% benzocaine (Sigma) before surgery. All surgeries were performed using Olympus SZX10 microscopes. To initiate regeneration, limbs were amputated at mid-humerus level and the bone was trimmed back from the amputation plane to produce a flat amputation surface. Animals were allowed to regenerate at 20°C for the indicated
FIGURE 7  Salamander-Eci protocol applied to study proliferation in axolotl limb regeneration. A, Representative snapshots of 3D reconstructions of axolotl limbs at the indicated times post-amputation. EdU (cyan) labels proliferating cells and Sytox green (grey) provides nuclear counterstaining. Scale bars: 500 μm (control and regenerated samples), 300 μm (all other time points). B, Schematic of the experimental procedure. C, The number of EdU cells in the area distal to the amputation plane, A, as well as in the area 300 μm proximal to the amputation plane, B, was quantified using Imaris software (n = 5). Statistical analysis: the number of EdU cells/mm$^3$ was compared to the control for every timepoint through one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison, revealing a significant effect of the regeneration time points $F(8, 61) = 2.850, P = .0093 (**). Tukey’s multiple comparison resulting P values: MB vs Control $P = .0354$, LB vs Control $P = .0002$, Palette vs Control $P = .0002$, DO vs Control $P = .0003$. The number of EdU cells was measured in the region A and B of the blastema per time-point and compared using two-way ANOVA. The interaction between the time points and the area of the blastema was not significant $F(8, 52) = 1.1, P = .3776$. 
times and euthanized by overdose of anesthesia prior to sample collection.

3.2 | EdU injection

EdU was dissolved in DMSO at 2.5 mg/mL. For smaller animals (<4 cm snout-tail-length, STL), EdU was delivered by intraperitoneal injection at 2.5 μL/g animal weight. For larger animals (>4 cm STL), EdU was delivered by intravenous injection at 1.5 μL/g animal weight. Glass capillaries (Harvard Apparatus) for injections were prepared using a P-97 Micropipette Puller (Sutter Instruments). Following a 1 hour EdU pulse, animals were euthanized by anesthesia overdose and samples fixed in 4% PFA overnight.

NB. The pulse length depends on the research requirements. For example, in order to take a snapshot of actively proliferating cells at a given time point, a 1 hour EdU pulse is recommended for animals between 3 and 7 cm STL.

3.3 | Sample collection and fixation

Fixative was prepared on the same day as sample collection by dissolving PFA to 4% (wt/vol) with PBS under constant heating in a water bath at 55°C. Once dissolved, PFA solution was cooled down to 4°C prior to use. Animals were anesthetized in 0.03% benzocaine solution, and samples collected and fixed in 4% PFA overnight. Consequently, the fixative needs time to dissolve, and therefore should be prepared at least 4 hours ahead of sample collection. To avoid over-fixation, it is recommended to keep the samples in fixative for under 1 day.

3.4 | Buffer preparation

Permeabilization buffer: 0.3 M Glycine, 2% Triton X-100 (vol/vol), 20% DMSO (vol/vol), in PBS.

Blocking buffer: 1.5% goat serum (vol/vol), 10% DMSO, 2% Triton X-100, in PBS.

Washing buffer: 2% Tween-20, 1% heparin 10 mg/mL, in PBS.

NB. Goat serum can be aliquoted and stored at −20°C. Once thawed, aliquots can be reused if stored at 4°C and supplemented with 0.1% (wt/vol) sodium azide.

3.5 | Permeabilization

After fixation, samples were washed twice in PBS for 15 minutes, and then transferred into permeabilization buffer for 1 to 3 days at room temperature.

NB. The permeabilization time required for thorough antibody diffusion varies according to sample size: 1 day is sufficient for small larvae (<1 cm) or small limbs/blastemas (<0.5 cm), whereas a more extensive permeabilization (3 days) is recommended for large samples, such as livers or spleens from >5 cm axolotls, juvenile axolotls (>2 cm) or large limbs (>1 cm).

3.6 | EdU click-it reaction

Only if EdU staining is required. Samples were washed twice in PBS for 5 minutes. EdU Click-it Plus reaction cocktail (ThermoFisher, C10339) was then prepared fresh according to Table 1 and used within 15 minutes of preparation. Samples were incubated for 6 hours at RT with gentle shaking whilst protected from light, and subsequently washed extensively in washing buffer 15 to 30 minutes at RT (four times) followed by an overnight wash.

NB. It is important to add the ingredients in the order listed in the table, otherwise, the reaction will not proceed optimally. Use the Click-it Plus reaction cocktail within 15 minutes of preparation.

→ From this stage onwards, all steps must be performed protected from light.

3.7 | Immunolabeling protocol

For immunostaining, samples were first incubated twice in blocking solution for 3 hours at RT, and subsequently incubated with primary antibody solution diluted in washing buffer for 1 to 3 days at RT (tested dilutions for antibodies are provided in Table 2). Samples were then soaked extensively for at least 30 minutes (four times) in washing buffer, followed by an overnight wash in washing buffer, and then incubated in secondary antibody diluted in washing buffer for 4 to 6 hours at RT (at dilutions provided in Table 3). Finally, samples were washed twice for 15 minutes in washing buffer and counter-stained with Sytox Green (1:2500 in washing buffer) for 3 hours at RT prior to dehydration and clearing (Tables 2 and 3).

NB. For primary antibody staining, 1 overnight incubation is sufficient for small samples (larvae < 1 cm or small

| Component | Total 1 mL | Total 3 mL |
|-----------|-----------|-----------|
| 1× Reaction buffer | 860 μL | 2580 μL |
| Copper protectant | 40 μL | 120 μL |
| AlexaFluor azide | 2.5 μL | 7.5 μL |
| 1× Reaction buffer additive | 100 μL | 300 μL |

TABLE 1 Click-it EdU reaction components, recommended volumes
limbs, blastemas), whereas an extended incubation—3 days—is required for larger samples, such as liver and spleen from >5 cm axolotls, large larvae (>2 cm) or large limbs (>1 cm).

### 3.8 Dehydration and clearing

**Optional:** If the sample is too small or fragile, agarose embedding prior to the dehydration and clearing process can aid in handling. Prepare agarose at 2%–4% (wt/vol) and dissolve it in either water or PBS.

Small samples were dehydrated with an ethanol dilution series at 4°C: 30% ethanol (in dH2O) for 2 hours, 50% ethanol for 2 hours, 70% ethanol for 2 hours, followed by incubation in 100% ethanol overnight. Larger samples and agarose-embedded samples were dehydrated with a more extensive dehydration series in 30%, 50%, 70% and 100% all overnight. Subsequently, samples were cleared by incubating in Eci for at least 1 hour with shaking (NB. Eci exists as a solid at 4°C, and hence it is necessary to thaw Eci at room temperature before use), until they became visually transparent. Eci-cleared samples were then stored in Eci at room temperature in the dark until imaging (NB. under these storage conditions Alexa Fluor- and nuclear staining-derived fluorescence is stable for up to 6 months).

### 3.9 Imaging and image processing

Cleared samples were imaged using a light-sheet microscope (Ultramicroscope Olympus MVX10, LaVision Biotec) and ImSpector Pro software (LaVision Biotec). This consisted of a single plane illumination microscope equipped with objective Olympus MVPLAPO 2x/0.5 coupled to a sCMOS camera (Andor Neo, camera pixel 2560 × 2160; pixel size 6.5 × 6.5 μm²). A correction lens was used in front of the sCMOS camera for chromatic aberrations. Images and videos were processed using Image J to carry out maximum projections of the Z plane. Imaris software (Bitplane) was used for volumetric analysis, video generation and snapshots from 3D projections and videos.

### 3.10 Quantification and statistics

The quantification of positive Edu cells was done by combining background subtraction using FIJI coupled with Imaris Bitplane for cell segmentation and volume determination. The number of EdU⁺ cells was quantified using the Spots tool (10 μm diameter) and volume was calculated using the Surfaces tool of Imaris (Bitplane). Quantification of mean voxel intensity was measured with Imaris Bitplane by selecting the area of the organ using the Surfaces tool and extracting the fluorescence information (Movies S4 and S5). Note that within this automated pipeline it is not possible to correct for false positives or negatives without resorting to manual curation. A detailed document explaining the script and usage, the FIJI script for pre-processing spleen images, the license file and a README file with the workflow details is publicly available at the MPI CBG Scientific Computing Gitlab repository (https://dx.doi.org/21.11101/0000-0007-E7A3-C). The analysis of signal intensity vs tissue depth was done by measuring the mean gray value in a region.
of interest (ROI) in every stack using FIJI. Mean gray values were plotted again normalized tissue depth (0-1).

Statistical analysis was performed using GraphPad Prism. If not otherwise stated, one-way analysis of variance was used for all experiments with post hoc Dunnet post-test to determine statistical differences between experimental and control groups. *P < .05, **P < .01, ***P < .001, ****P < .0001 were considered significant. Data values represent mean ± SD unless indicated otherwise.

ACKNOWLEDGMENTS
We thank all members of the Yun Lab for critical comments, the CMCB Light Microscopy Facility for microscopy support, Gayathri Nadar and the MPI-CBG Scientific Computing Facility for help with image analysis, and Beate Gruhl and Anja Wagner for animal care and breeding. Cristina Subiran Adrados was supported by a CRTD MSc stipend. Lizbeth Airais Bolaños Castro was supported by a DAAD PhD Scholarship. Maximina Yun was supported by the Center for Regenerative Therapies Dresden (DFG FZ 111, DFG EXC 168). The Miltenyi Biotec Ultramicroscope II belongs to CMCB microscopy facility and is partially financed by funds from the European Regional Development Fund (EFRE). Open Open access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS
Cristina Subiran Adrados: Data curation; formal analysis; investigation; methodology; visualization; writing-original draft. Qinghao Yu: Data curation; formal analysis; investigation; methodology; writing-original draft. Lizbeth Airais Bolaños Castro: Investigation; methodology; writing-review and editing. Luis Alberto Rodriguez Cabrera: Conceptualization; data curation; methodology. Maximina Hee Yun: Conceptualization; funding acquisition; project administration; supervision; writing-original draft; writing-review and editing.

CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

ORCID
Maximina Hee Yun https://orcid.org/0000-0001-9019-2453

REFERENCES
1. Oh SW, Harris JA, Ng L, et al. A mesoscale connectome of the mouse brain. Nature. 2014;508(7495):207-214. https://doi.org/10.1038/nature13186.
2. Toga AW, Goldkorn A, Ambach K, Chao K, Quinn BC, Yao P. Postmortem cryosectioning as an anatomic reference for human brain mapping. Comput Med Imaging Graph. 1997;21(2):131-141. https://doi.org/10.1016/s0895-6111(96)00072-9.
3. Taqi SA, Sami SA, Sami LB, Zaki SA. A review of artifacts in histopathology. J Oral Maxillofac Pathol. 2018;22(2):279. https://doi.org/10.4103/jomfp.JOMFP_125_15.
4. Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell. 2014;159(4):896-910. https://doi.org/10.1016/j.cell.2014.10.010.
5. Renier N, Adams EL, Kirst C, et al. Mapping of brain activity by automated volume analysis of immediate early genes. Cell. 2016;165(7):1789-1802. https://doi.org/10.1016/j.cell.2016.05.007.
6. Richardson DS, Lichtman JW. Clarifying tissue clearing. Cell. 2015;162(2):246-257. https://doi.org/10.1016/j.cell.2015.06.067.
7. Ueda HR, Erturk A, Chung K, et al. Tissue clearing and its applications in neuroscience. Nat Rev Neurosci. 2020;21(2):61-79. https://doi.org/10.1038/s41583-019-0250-1.
8. Chung K, Wallace J, Kim SY, et al. Structural and molecular interrogation of intact biological systems. Nature. 2013;497(7449):332-337. https://doi.org/10.1038/nature12107.
9. Yang B, Treweek JB, Kulkarni RP, et al. Single-cell phenotyping within transparent intact tissue through whole-body clearing. Cell. 2014;158(4):945-958. https://doi.org/10.1016/j.cell.2014.07.017.
10. Pende M, Vadiwala K, Schmidbaur H, et al. A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity. Sci Adv. 2020;6(22):eaba0365. https://doi.org/10.1126/sciadv.aba0365.
11. Masselink W, Reumann D, Murawala P, et al. Broad applicability of a streamlined ethyl cinnamate-based clearing procedure. Development. 2019;146(3):dev166884. https://doi.org/10.1242/dev.166884.
12. Klingberg A, Hasenberg A, Ludwig-Portugall I, et al. Fully automated evaluation of total glomerular number and capillary tuft size in nephritic kidneys using lightsheet microscopy. J Am Soc Nephrol. 2017;28(2):452-459. https://doi.org/10.1681/ASN.2016020232.
13. Hama H, Kurokawa H, Kawano H, et al. Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat Neurosci. 2011;14(11):1481-1488. https://doi.org/10.1038/nn.2928.
14. Ke MT, Fujimoto S, Imai T. SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nat Neurosci. 2013;16(8):1154-1161. https://doi.org/10.1038/nn.3447.
15. Gradinaru V, Treweek J, Overton K, Deisseroth K. Hydrogel-tissue chemistry: principles and applications. Annu Rev Biophys. 2018;47:355-376. https://doi.org/10.1146/annurev-biophysics-070317-032905.
16. Khattak S, Schuez M, Richter T, et al. Germline transgenic methods for tracking cells and testing gene function during regeneration in the axolotl. Stem Cell Reports. 2013;1(1):90-103. https://doi.org/10.1016/j.stemcr.2013.03.002.
17. Lopez D, Lin L, Monaghan JR, et al. Mapping hematopoiesis in a fully regenerative vertebrate: the axolotl. Blood. 2014;124(8):1232-1241. https://doi.org/10.1182/blood-2013-09-526970.
18. Leigh ND, Dunlap GS, Johnson K, et al. Transcriptomic landscape of the blastema niche in regenerating adult axolotl limbs at single-cell resolution. *Nat Commun*. 2018;9(1):5153. https://doi.org/10.1038/s41467-018-07604-0.

19. Currie JD, Kawaguchi A, Traspas RM, Schuez M, Chara O, Tanaka EM. Live imaging of axolotl digit regeneration reveals spatiotemporal choreography of diverse connective tissue progenitor pools. *Dev Cell*. 2016;39(4):411-423. https://doi.org/10.1016/j.devcel.2016.10.013.

20. Khattak S, Murawala P, Andreas H, et al. Optimized axolotl (*Ambystoma mexicanum*) husbandry, breeding, metamorphosis, transgenesis and tamoxifen-mediated recombination. *Nat Protoc*. 2014;9(3):529-540. https://doi.org/10.1038/nprot.2014.040.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

*How to cite this article:* Subiran Adrados C, Yu Q, Bolaños Castro LA, Rodríguez Cabrera LA, Yun MH. Salamander-Eci: An optical clearing protocol for the three-dimensional exploration of regeneration. *Developmental Dynamics*. 2021;250:902–915. https://doi.org/10.1002/dvdy.264