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Labeling of single cells in the central nervous system of *Drosophila melanogaster* --Manuscript Draft--

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Labeling of single cells in the CNS of *Drosophila melanogaster*

Dear Dr. Standen,

thanks a lot for your mail from July 19th.

In the revised version of our manuscript we have addressed all comments of the referees (as listed in a separate file).

Many thanks for your efforts and best regards

Christof Rickert and Gerd Technau



Labeling of single cells in the central nervous system of *Drosophila melanogaster*

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Short Abstract

We present a technique for labeling single neurons in the central nervous system (CNS) of *Drosophila* embryos, which allows the analysis of neuronal morphology by either transmitted light or confocal microscopy.

Long Abstract

In this article we describe how to individually label neurons in the embryonic CNS of *Drosophila melanogaster* by juxtacellular injection of the lipophilic fluorescent membrane marker Dil. This method allows the visualization of neuronal cell morphology in great detail. It is possible to label any cell in the CNS: cell bodies of target neurons are visualized under DIC optics or by expression of a fluorescent genetic marker such as GFP. After labeling, the Dil can be transformed into a permanent brown stain by photoconversion to allow visualization of cell morphology with transmitted light and DIC optics. Alternatively, the Dil-labeled cells can be observed directly with confocal microscopy, enabling genetically introduced fluorescent reporter proteins to be colocalised. The technique can be used in any animal, irrespective of genotype, making it possible to analyze mutant phenotypes at single cell resolution.

INTRODUCTION

Knowledge of neuronal morphology at the level of individual cells is a key prerequisite for understanding neuronal connectivity and CNS function. Thus, from the earliest days of neuroscience, researchers have sought to develop single cell labeling techniques (see

(Lanciego and Wouterlood, 2011) for a historical treatment of this issue). Classical methods such as Golgi staining provide excellent resolution of neuronal morphology but are not suitable if one seeks to label a particular type of neuron in a directed way, as staining occurs in a random fashion. The development of methods for staining single cells by intracellular or juxtacellular injection of dyes from a microelectrode addressed the requirement of specific labeling.

The application of single neuron dye injection to *Drosophila* presented a major challenge, because of the small size of both the organism and its neurons. Nonetheless, single neuron staining of embryonic *Drosophila* neurons was achieved in the mid 1980s in the laboratory of Corey Goodman (Thomas et al., 1984). While the method has been eminently useful and has provided some key insights into mechanisms for neuronal development in *Drosophila* over the last 20-30 years (e.g. (Broadie et al., 1993; Matthes et al., 1995), many workers have shied away from it, largely because of its technical demands.

The availability in more recent years of genetic techniques for neuronal labeling in *Drosophila* has also contributed to the unpopularity of single neuron dye injection. GAL4-directed expression of membrane-targeted GFP constructs can provide excellent resolution of neural morphology (Brand and Perrimon, 1993; Williams et al., 2000). However, this method has certain limitations: the often-unavoidable expression of GFP in multiple cells can obscure the structure of individual neurons and a GAL4 driver line may not be available to drive expression in a particular neuron of interest. The MARCM (Mosaic Analysis with a Repressible Cell Marker) method (Lee and Luo, 1999) can provide labeling of essentially any neuron at the individual cell level, but cannot be successfully used in the embryo and early larva because of the slow turnover of the GAL80 protein.

Given these limitations of genetic labeling, we believe that single neuron dye injection in the *Drosophila* embryo remains a valuable technique and deserves broader application. To promote this goal, we provide here a detailed description of the method. An illustration of its power is provided by our recent account of the morphology of the complete set of interneurons in abdominal neuromeres of the late *Drosophila* embryo (Rickert et al., 2011). This study, which revealed both the morphological variability of individual neuronal cell types and the principles of neuromere organization in the CNS of the embryo, would not have been possible with any other currently available labeling method.

PROTOCOL

We have used two slightly different variants of the single neuron labeling method in our laboratories. The differences relate to the embryo collection, dechorionisation, devitellinisation and embryo filleting steps. Figure 1 gives an overview of the common and diverging steps of the variants.

1) Preparation of micro-needles for embryo dissection and micropipettes for dye injection

1.1) Glass needles for embryo dissection are drawn from glass capillaries (1 mm diameter and 0.1 mm wall thickness) with a Sutter puller (Science Instruments) or comparable equipment. Alternatively, an electrolytically sharpened 0.15mm tungsten wire mounted in a

needle holder is used for dissections. Instructions for electrolytic sharpening are given in (Levick, 1972)).

1.2) Dye Injection micropipettes are drawn from thin-walled glass capillaries with inner filaments (Science Products; GB 100 TF 8P) with a Sutter puller (Science Instruments) or comparable equipment. The micropipette tip needs to be sharp, with a gradual, uniform taper of the shank to aid passage through the tissues of the embryo. The desired micropipette is the “High Resistance Microelectrode, Sharp and Long” variety, as described on p.20 of the Sutter Instrument Pipette Cookbook manual (http://www.sutter.com/contact/faqs/pipette_cookbook.pdf). Generally, micropipettes are pulled shortly before injections are performed to ensure their tips remain sharp and clean.

2) Collection, Mounting and Dissection of Embryos

2.1) Embryo Collection. We have successfully used the neuron injection method described here in embryos from stages 12 to 17 (Campos-Ortega and Hartenstein, 1985). Embryos progressively develop a cuticle during stage 17 and become increasingly difficult to dissect. Two alternative approaches are available to obtain embryos at a particular developmental stage.

2.1A) Allow flies to lay eggs overnight on apple juice-agar plates coated with yeast paste. Adjust the temperature of egg collection to suit the planned time of injection the next day. Collect all eggs the next day and select embryos at the desired stage using morphological criteria (Campos-Ortega and Hartenstein, 1985).

2.1B) Allow flies to lay on agar plates as above, but swap out the agar plate with a fresh one every 1.5 hours at 25°C. Incubate each plate for a defined time period, until embryos have reached the developmental stage required.

2.2A) Chemical Dechoriation. Use a metal spatula to scrape embryos off the agar and transfer them into a glass vessel (e.g. petri dish or cavity block) containing approximately 10ml Drosophila Ringer or Phosphate buffered saline (PBS) solution. Add a few drops of concentrated bleach and agitate for a few minutes on a rotary platform until the chorion sloughs off the embryos. Wash embryos in several changes of Ringer/PBS until there is no smell of residual bleach. During these washes, ensure that the embryos do not come into contact with the liquid surface. Otherwise, they will float and become difficult to submerge for later manipulation.

Alternatively, chemical dechoriation may be carried out using the basket technique described by (Featherstone et al., 2009).

2.2B) Mechanical dechoriation (see Figure 2, steps 1-4). Transfer embryos from the agar plate to a slide covered with double-sided sticky tape. Avoid transferring agar with the embryos as it will interfere with their adhesion to the tape. Allow embryos to dry for 5 to 10 minutes until the chorion gets slightly brittle. (While waiting, coat the coverslip needed for later devitellinisation with glue - see 2.3B below). Touch each embryo gently with a needle. The chorion will split open and the embryo will stick to the needle. Transfer embryos immediately to an agar block to prevent drying and align about 10 of them in a row, with their ventral sides facing up.

Devitellinisation and filleting are done manually using either of the two methods described in 2.3A and 2.3B.

2.3A) Transfer a single dechorionated embryo of the appropriate developmental stage from the dish of Ringer solution to several drops of Ringer in a silicone dam on a pre-prepared microscope slide. (Make a 3cm square dam by smearing a thin layer of silicone sealant on a microscope slide, then add a drop of 0.01% poly-L-lysine solution to the center of the dam. Allow the silicone to cure for 24 hours.) Transfer the embryo with a glass Pasteur pipette, ensuring that the embryo remains beneath the surface of the Ringer during the transfer.

Brace the posterior end of the embryo with a pair of fine forceps, while gently squeezing the embryo with a pair of fine iridectomy scissors, about a quarter the way back from its anterior end. Do not cut right through the embryo. The aim is to just crack open the vitelline membrane while causing minimal damage to the embryo. With the forceps still in position, use a tungsten needle to ease the embryo out of the opened membrane and position it ventral side down on the slide. The embryo should stick to the poly-lysine coat.

Fillet the embryo with a sharpened tungsten needle. Gently perforate, then tear the body wall along the dorsal midline. Using the needle, push down on the body wall so that it sticks to the slide. To prevent damage to the body wall, push the body wall with the gut interposed between it and the needle. Then use the needle to tear through the gut at its anterior and posterior ends and lift it away. If desired, additional embryos may be transferred to the same slide, devitellinised and filleted, taking care not to damage other embryos already on the slide.

2.3B) Coat a 24 x 60 mm coverslip with heptane glue (see Tab.1) by placing a small drop of glue in the center of the slide and spreading it with a 18 x 18 mm coverslip to a very thin film. Place the coverslip on a microscope slide and make a frame of one-sided adhesive tape around its edges. Cut away excess agar from the block holding the row of 10 embryos, gently touch the embryos with the coverslip heptane glue coverslip. They should now adhere to the coverslip with their ventral sides facing down. Add a generous amount of PBS to cover the embryos (see Figure 2, steps 4-6).

Penetrate the vitelline membrane with a glass or tungsten needle on the dorsal side of each embryo near its posterior end. Tear open the membrane along the dorsal midline and drag the embryo out of the membrane. Orient the embryo ventral side down elsewhere on the heptane glue substrate and fillet it using the same method described in 2.3A) (see Figure 2, steps 7-8). Since several embryos will be filleted on the same slide, it is helpful to either be very accurate with their orientation or make a drawing of their orientation on the slide.

2.4) After filleting, embryos may be lightly fixed for 10-15 minutes in 7.4 % formaldehyde in PBS, followed by 4 washes in PBS. Extreme care must be taken not to bring the embryo into contact with the surface of the solution during this process, as the surface tension forces will destroy the embryo. This pre-fixation step is not strictly necessary, but is useful to prevent contraction of the body wall muscles in late stage embryos and to help stabilize embryonic tissues at young stages. Keep in mind that fixation does make the tissues of the embryo more opaque and so somewhat compromises image quality under DIC observation.

3) Filling of injection micropipettes

Half fill a 0.5ml Eppendorf microfuge tube with a 0.1% solution of the carbocyanine dye Dil (Molecular Probes, Eugene, OR) in 100% ethanol (be sure to use 'dry' absolute EtOH to avoid Dil precipitation). Make a narrow hole in the lid of the tube and insert the blunt end of the micropipette through the hole in the lid. Allow the Dil to ascend up the filament for at least 5 min. (Always cover the hole in the lid when not filling a micropipette to avoid evaporation of ethanol).

Equipment required for neuron dye injection (Fig. 3).

Microscope. A fixed stage microscope must be used for neuron dye injection to avoid vertical movement of the embryo during focussing. Any such movement would displace the pipette after it has come into contact with the embryo. We have found both the Zeiss Axioskop FS and the Olympus AX50/BX50 fixed stage models to be well suited for this purpose. The microscope should be equipped with transmitted light DIC optics for visualization of neurons before staining. The microscope should also be an upright rather than an inverted model. With an upright microscope, the tip of the micropipette is on the same side of the embryo as the viewing objective, whereas with an inverted microscope the two are on opposite sides of the embryo. The latter arrangement compromises the quality of DIC optics. The microscope should be set up for fluorescence microscopy, with a suitable filter set for Dil observation. Since Dil has a comparably broad spectrum with excitation and emission maxima at 549 and 565 nm many filter sets in this range will work, e.g. those for Alexa 568, Cy3, Rhodamine, Texas Red or TRITC. Although it is convenient to fit an electronically controlled shutter in the path of the fluorescence light source, to allow operation of the shutter without hand contact with the microscope, we also effectively labeled on a setup which was only equipped with a manual shutter. Finally, a high magnification, high numerical aperture water immersion objective is required for observation during injection. This objective should be designed for use without a coverslip. We have successfully used the Zeiss Achromplan 100x/1.0W, Olympus LUMPlan FL 100x/1.0W, and Olympus LUMPlan FL/IR 60x/0.9 W objectives.

A **micromanipulator** is required to position and move the micropipette during neuron dye injection. We have used both the Leica micromanipulator and a stage-mounted Narashige 3-axis hydraulic micromanipulator.

An **intracellular DC amplifier**, with the facility for current injection, is required for iontophoretic injection of Dil from the micropipette.

4) Procedure for dye injection

4.1) Place the slide with the mounted embryo(s) on the stage of the injection microscope (Figure 3). Use a 10x objective to locate an embryo and bring it into the center of the field of view.

4.2) Swing the high power objective into the viewing position and bring the embryo into focus. Locate the cell of interest under DIC optics (or using fluorescence if the target cell expresses GFP) and bring it into the centre of the field of view. Ensure that the field diaphragm of the microscope is opened just to the edge of the field of view, not beyond. A narrow illumination beam will aid positioning of the micropipette (see step 4.4 below). Raise

the objective until it is as high as possible above the embryo while still remaining in contact with the Ringer/PBS solution.

4.2) Place the bath electrode for the DC amplifier into the PBS well clear of the embryo and the path of the micropipette.

4.3) Insert the injection micropipette into its holder, which has been filled with a 0.1M LiCl solution. Attach the micropipette holder to the micromanipulator. Using the micromanipulator coarse controls, bring the micropipette into the space between the tip of the objective and the embryo. Ensure that it is well above the level of the embryo. Because of the short working distance of the high power objective, the micropipette will have to be held at a shallow angle in order to bring it into focus (see Figure 3). You will have to establish the appropriate angle by trial and error in the first instance. If the angle is too steep, you will not be able to get the micropipette tip into focus. If it is too shallow, the micropipette shaft will foul against the wall of the dam holding the bathing solution in place.

4.4) Center the tip of the micropipette in the field of view. To achieve this, bring your eyes to the level of the embryo and move the micropipette back and forth in the Y-axis of the microscope stage. When its tip crosses the light path, you will see the bright reflection of the light rays from it. Move the tip of the micropipette forward in the X-axis so that it overshoots the light beam. It will be easier to locate the micropipette in the small field of view of the high power objective if you are looking for its shaft rather than its tip. Now look through the microscope eyepieces and gradually lower the high power objective until the shaft of the micropipette comes into focus. Moving the micropipette back and forth in the Y-axis with the micromanipulator controls helps to locate it, as it gradually comes into focus. When the shaft is in focus, move the micropipette in the X-axis until its tip lies in the center of the field of view. At this stage the micropipette tip should be well above the level of the embryo. Switch to fluorescence illumination and examine the tip to check for leakage of Dil. Adjust the DC current to prevent any Dil crystal from forming at the tip.

4.5) Using the micromanipulator controls, lower the micropipette in the Z-axis. Bring it back into focus with the microscope focus control. Progressively lower the micropipette down towards the embryo in this way. Initially, you can do this with the coarse Z-axis controls of the micromanipulator and microscope. However, as the embryo comes into focus you should switch to the fine control knobs.

4.6) When the surface of the embryo comes into focus, move the tip of the micropipette towards the edge of the field of view, in the direction of the micromanipulator. Focus on the cell to be injected and check that it lies at the center of the field of view. Refocus on the micropipette tip and move it in the Y-axis until it lies at the same level in the Y-axis as the cell. Move the tip of the micropipette towards the cell in the X-axis as you lower it in the Z-axis. If using a Leica micromanipulator, you will probably find that the microscope stage controls give you finer control of movement in the X-axis than the micromanipulator controls, so switch to the stage control as the tip gets close to the cell.

4.7) Bring the micropipette tip into contact with the cell and make a depression on its surface. Pass several nanoamps of depolarizing current for a few seconds. You should see a small crystal of Dil forming on the cell. To confirm that the cell has been labeled, briefly open

the shutter to illuminate the embryo with fluorescent light, then switch back to DIC. If the body of the cell of interest shows signs of labeling, apply current for several more seconds. Turn off the current and quickly pull the embryo away from the micropipette in the X-axis using the microscope stage control. Then withdraw the micropipette from the solution.

If no Dil labeling is evident, the micropipette may be blocked and will require replacement with a fresh micropipette. You may find that the tip of the micropipette has snagged other tissues en route to the cell of interest and this tissue is stained rather than the cell. In this case, try withdrawing the micropipette slightly, and re-approach the cell. It may be necessary to replace the micropipette and try again.

4.8) If desired, multiple cells can be individually labeled in the same embryo by repeating steps 4.4-4.7 above.

4.9) Remove most of the solution in the injection chamber, then fix the embryos by adding 7.4 % formaldehyde/PBS for 10 minutes followed by 4 washes with PBS. The embryo is then left at room temperature for at least 4 hours (or overnight at 4°C) in a dark, humid chamber (to avoid bleaching or dry falling) to allow the Dil to diffuse throughout neuronal processes. At this stage, the Dil-labeled cells may be either photoconverted or viewed directly in the confocal microscope.

5) Photoconversion for examination using DIC optics

The principle of photoconversion is the (photo-)oxidation of DAB by the fluorescent light emitted by the stained cell during illumination with the appropriate wavelength. This means bright cells are photoconverted in shorter time compared to weakly stained cells. If multiple cells labeled in one specimen differ too much regarding their brightness this can lead to difficulties in photoconverting all of them in same quality (see Fig. 4C): in this case one may have to decide on a compromise between neglecting the weaker cells (using short illumination) or accepting that the brighter cells begin to swell (using longer illumination). If all cells are too weakly labeled (thus needing very long illumination), background caused by endogenous peroxidases can become a problem.

Since DAB is toxic it should be handled with care. Waste can be 'deactivated' with 7% chlorine bleaching.

5.1) Photoconversion must be carried out for each embryo individually. In cases where only one embryo is injected per slide, the PBS solution covering the embryo is replaced with a DAB solution (3mg DAB/ml Tris buffer), the slide placed on a fluorescence microscope and the filled cell viewed with a 100x objective. (Using an upright microscope the objective dips into the DAB solution and should be cleaned several times with water after the photoconversion procedure is finished; this can be avoided if an inverted microscope is used). A Cy3 filter set and a 100W Hg lamp are used and the cell exposed to the red excitation wavelengths until a brown DAB reaction product becomes evident in the labeled neuron (check periodically by switching to bright field illumination). The time taken for optimal DAB staining is variable, but requires exposure to the excitation light beyond the stage where the fluorescence has completely faded. After photoconversion is complete, wash the preparation several times with PBS. Replace the PBS with a drop of 70% glycerol,

scrape away the silicone with a scalpel blade, replace with a ring of Vaseline and add a coverslip.

5.2) When multiple embryos have been filled on the one slide, transfer each embryo to a small drop of PBS on a separate 24 x 60 mm coverslip with the ventral side of the embryo facing the glass. Place a frame of adhesive tape around each embryo to create a well and cover the preparation with PBS. Keep the slides in a moist chamber before photoconversion to prevent them from drying out. Exchange the PBS covering the embryo with DAB solution. Immediately place the slide onto an inverted microscope equipped with a 100W Hg lamp and use a Cy3 filter set to excite the Dil, using a x 50 objective. After photoconversion, transfer the embryo to a fresh slide in a drop of 70% glycerol, add a coverslip and seal with nail polish.

6) Examination by Confocal Microscopy

6.1) After step 4.9, transfer embryos to a small drop of PBS on a fresh 24 x 60 mm coverslip. We achieve optimal optics by mounting the flattened embryos with the dorsal side towards the coverslip (leaving only a small amount of PBS between the coverslip and embryo).

6.2) Place a frame of adhesive tape around the embryo and enlarge the PBS drop to fill the frame when covered with a slide. Place a slide on it carefully and fix it with nail polish. Filled neurons should be examined on a confocal (or fluorescence) microscope as soon as possible.

REPRESENTATIVE RESULTS

Figure 4 collects typical results of the technique, we describe here. Fig. 4A shows an example of a Dil filled single interneuron that was flawlessly photoconverted. It nicely demonstrates the amount of detail these preparations offer. When viewed under DIC optics the spatial context of the labeled cell within the non-labeled surrounding tissue becomes visible, e.g. the position of the cell body within the cortex and of the fiber projection within the neuropil.

Fig.4B is a case where the dye drop was a little too big resulting in several neighbouring cells becoming labeled simultaneously. This often makes it difficult to relate individual projections to distinct cell bodies. It also shows that when directly viewed under the fluorescent microscope (no photoconversion) background resolution is much lower.

The specimen in Fig. 4C may serve as a representative for multiple cells being individually labeled in neighboring segments. Upon photoconversion the preparation was subsequently stained with an antibody against Fas2 (Grenningloh et al., 1991) following standard protocols (Patel, 1994) to provide landmarks (fascicles) in the neuropile. Although in this preparation most cell bodies are nicely separated and allow reliable mapping of their projections it also demonstrates a side effect which may occur when the photoconversion period is prolonged (in order to also convert the weakly labeled cells): the more intensely labeled cells tend to get overstained and start to swell (compare with A).

Fig. 4D shows that also documentation with confocal microscopy provides detailed morphology of labeled cells. Dil labeling was performed in a strain that carried a GFP reporter construct. This may provide important information about the spatial context (and identity) of the dye filled cell within specific populations of neuronal or glial cell types (as defined by gene expression).

As a paradigm to evaluate morphological variability of an identified neuron we have chosen one of the Apterous positive neurons (Rickert et al., 2011). This neuron lies in a dorsal and medial position close to the neuropile (dAP, Figure 5A, Lundgren et al., 1995) separate from the other Apterous positive neurons and is therefore easily identifiable in apGal4-UASGFP animals. Figure 5B shows a dAP cell that was filled with Dil and photoconverted. The cell body lies at the level of the anterior commissure and shows an axon that first grows towards the midline and then turns anterior in a medial connective region. It has growth cone like swellings at the tip and the turning point. 19 labels of this cell, drawn as maximum projections from image stacks, are summarized in Figure 5C. Cell morphology becomes visible to great detail. Only few cells show growth cone like structures; six of the 19 cells also send a branch posterior that is always shorter than the anterior branch and may well be a transient feature. In figure 5D all 19 dAP cells labeled are stacked with each cell having only an opacity of 12%. This results in areas being darker the more cells share them. While the cell body varies more than one cell diameter around the centre position, the mediolateral position of the axon within the neuropile varies much less – when the neuropile width is divided into nine parts it resides always in one of the medial two positions.

DISCUSSION

One major advantage of *Drosophila* as a model system is that it allows analysis of development and function on the level of single cells. This is especially helpful regarding the nervous system, where the diversity of cell types is exceptionally high and the function and morphology of neighboring cells can be totally different.

The method we present here allows the labeling of individual neurons with a dye that can either be transformed into a permanent stain or examined directly by fluorescence microscopy. It reveals the morphology of neural processes in great detail (Figure 4). One of the major advantages of the method is that it enables the morphology of virtually any type of neuron in the CNS to be examined (for the embryonic ventral nerve cord, see Rickert et al., 2011; for the embryonic brain, see Kunz et al., 2012). In addition, it does not require complex combinations of genetic elements to be present in the embryo, as several of the genetic labeling techniques do. Dye injections may be performed into cells of animals that carry reporter constructs, thereby allowing individual cell morphologies to be visualised in the context of particular gene expression patterns (Figures 4D and 5) in either wild type or mutant backgrounds. We have also used the technique to monitor the dynamics of axon outgrowth in living embryonic neurons (Murray et al., 1998; Murray and Whittington, 1999).

The method can be readily adapted to label individual neurons in embryos of other organisms, provided the embryo is transparent enough to be examined under DIC illumination. We have used it to visualize neural morphology in a wide variety of arthropod embryos, including grasshoppers (Whittington and Seifert, 1984), centipedes (Whittington and Meier, 1991), crustaceans (Whittington et al., 1993) and silverfish (Whittington and Harris, 1996).

The major disadvantage of the technique lies in its technical difficulty. We trust that the current detailed account of the method will assist interested researchers in mastering it.

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DISCLOSURES: The authors declare that they have no competing financial interests

Figure Legends

Figure 1. Schematic overview of the steps of the neuron labeling method and its two variants.

Figure 2. Diagram illustrating the various steps in preparing *Drosophila* embryos for neuron dye injection, according to variant B.

Figure 3. Schematic representation of the setup for dye injection. The upper right insert is an enlarged view of the arrangement of the specimen, the injection micropipette and the bath electrode.

A: Micromanipulator, B: Fixed stage microscope. The micromanipulator is set at a shallow angle – approximately 10°. C: Micropipette holder with micropipette, D: Specimen on slide, E: Bath electrode fixed with plasticine, F: Connection to DC amplifier.

Figure 4. Examples of Dil-labeled interneurons in the ventral nerve cord of stage 17 embryos. Dorsal views, anterior is to left. Midline: dotted lines.

A) An example of a perfectly photoconverted single cell, showing a prominent contralateral axonal projection and ipsilateral dendritic fibers (arrowhead). DIC optics.

B) shows a preparation in which more than one cell was labeled – this can obscure the clear assignment of a single cell body to its projection.

In C) 8 cells have been labeled in 3 consecutive segments. In addition, in this preparation the photoconversion was followed by a standard protocol antibody staining against Fas2 as a landmark.

D) shows a single Dil-filled neuron documented with confocal microscopy in a fly strain carrying a GFP reporter construct.

Figure 5. Targeted Dil labeling (marked by GFP expression) reveals the range of morphological variability of an identified neuron (from Rickert et al. 2011).

A) The dAP cell (artificially coloured blue) can be unambiguously recognised within the *apterous*Gal4-UASGFP pattern in the abdomen (Lundgren et al. 1995). GFP expressing cells have been stained with an anti-GFP antibody. The midline is marked with dotted lines. ac = anterior commissure, pc = posterior commissure.

B) shows an example of a photoconverted Dil-filled dAP cell. The cell was identified prior to dye filling in an *apterous*Gal4-UASGFP embryo by its expression of GFP.

C) A gallery of 19 fills of this cell, drawn as maximum projections from image stacks. Dorsal views. Anterior is up. The neuropile is in light grey, the cortex area is in darker grey.

D) The 19 dAP cells are stacked with each cell having an opacity of 12%. The region occupied by most of the cells has the darkest shade of grey. The neuropile of all specimens (blue) was scaled to a similar width and the anterior commissures next to the cell were aligned so they overlapped. While the cell body varies more than one cell diameter around the central position, the mediolateral position of the axon within the neuropile shows less variation.

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Figure 1
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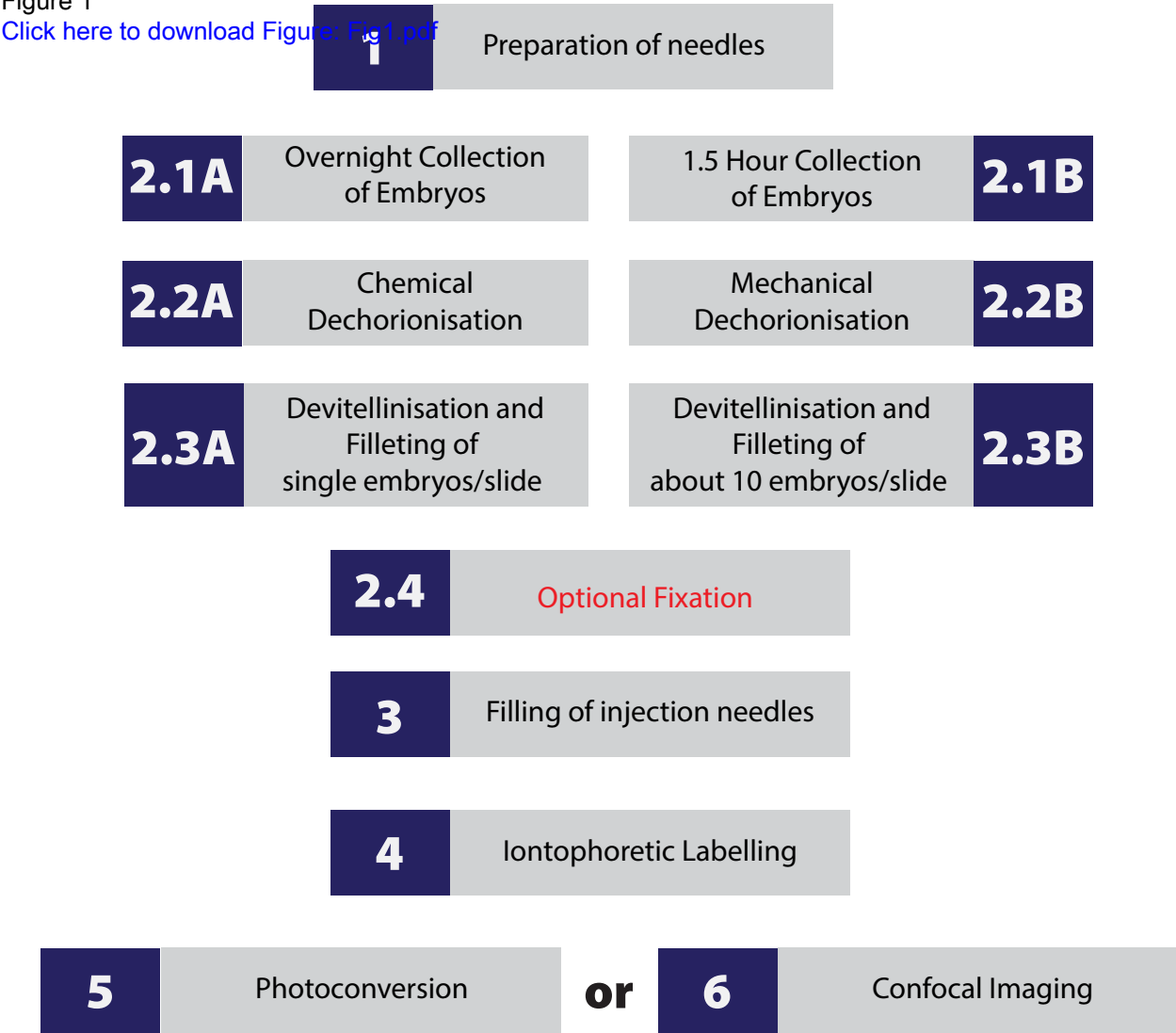


Figure 2
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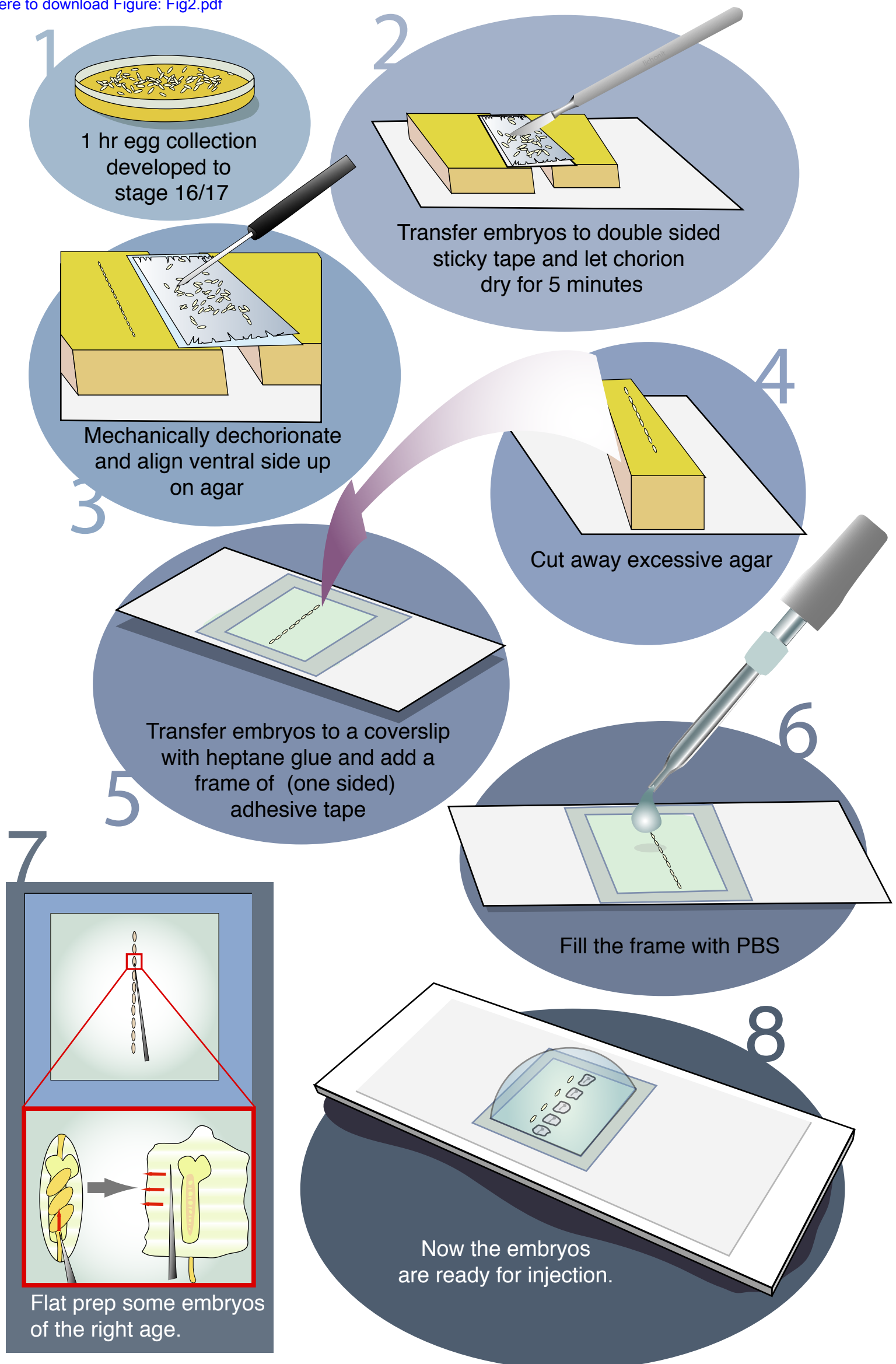


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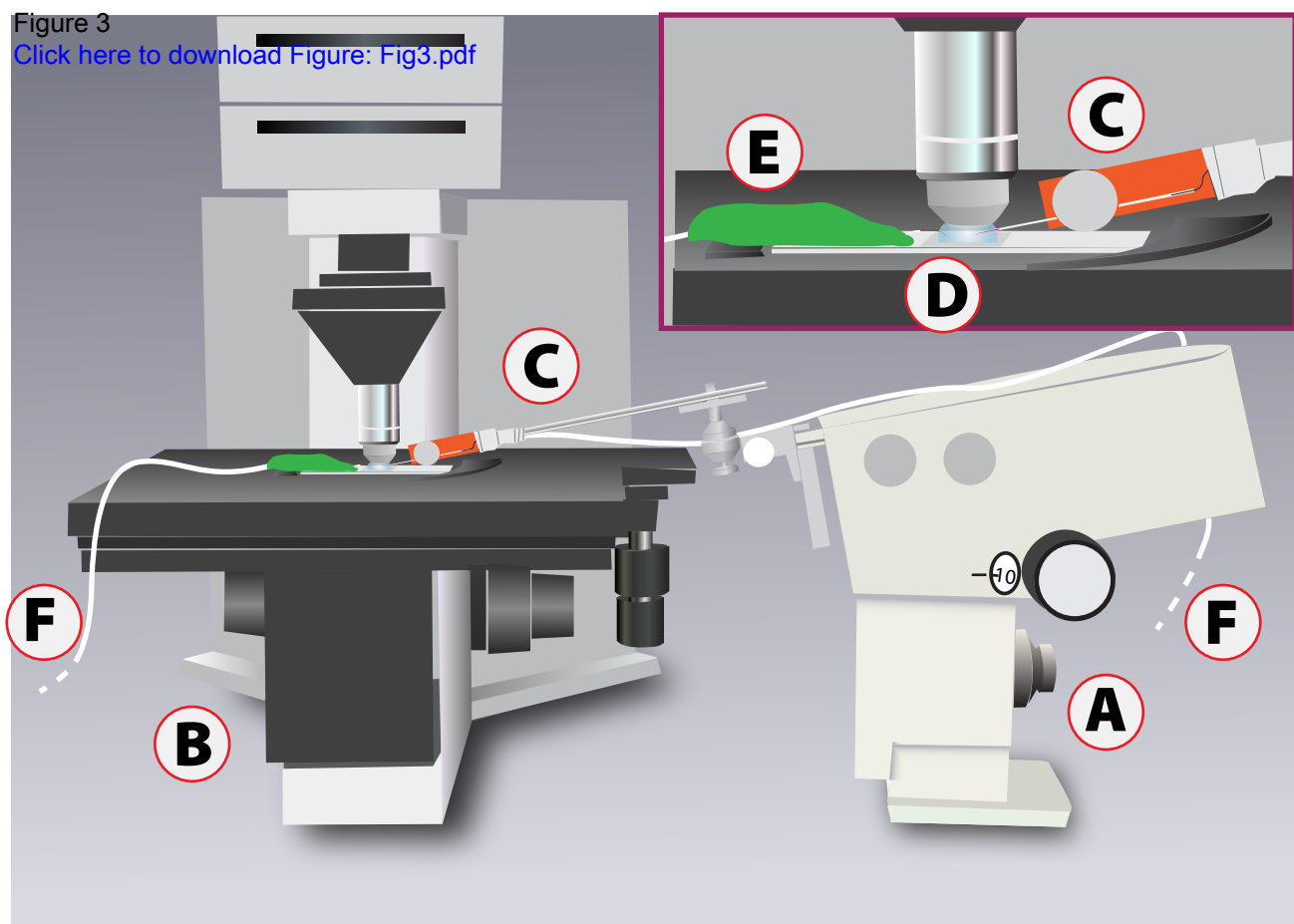
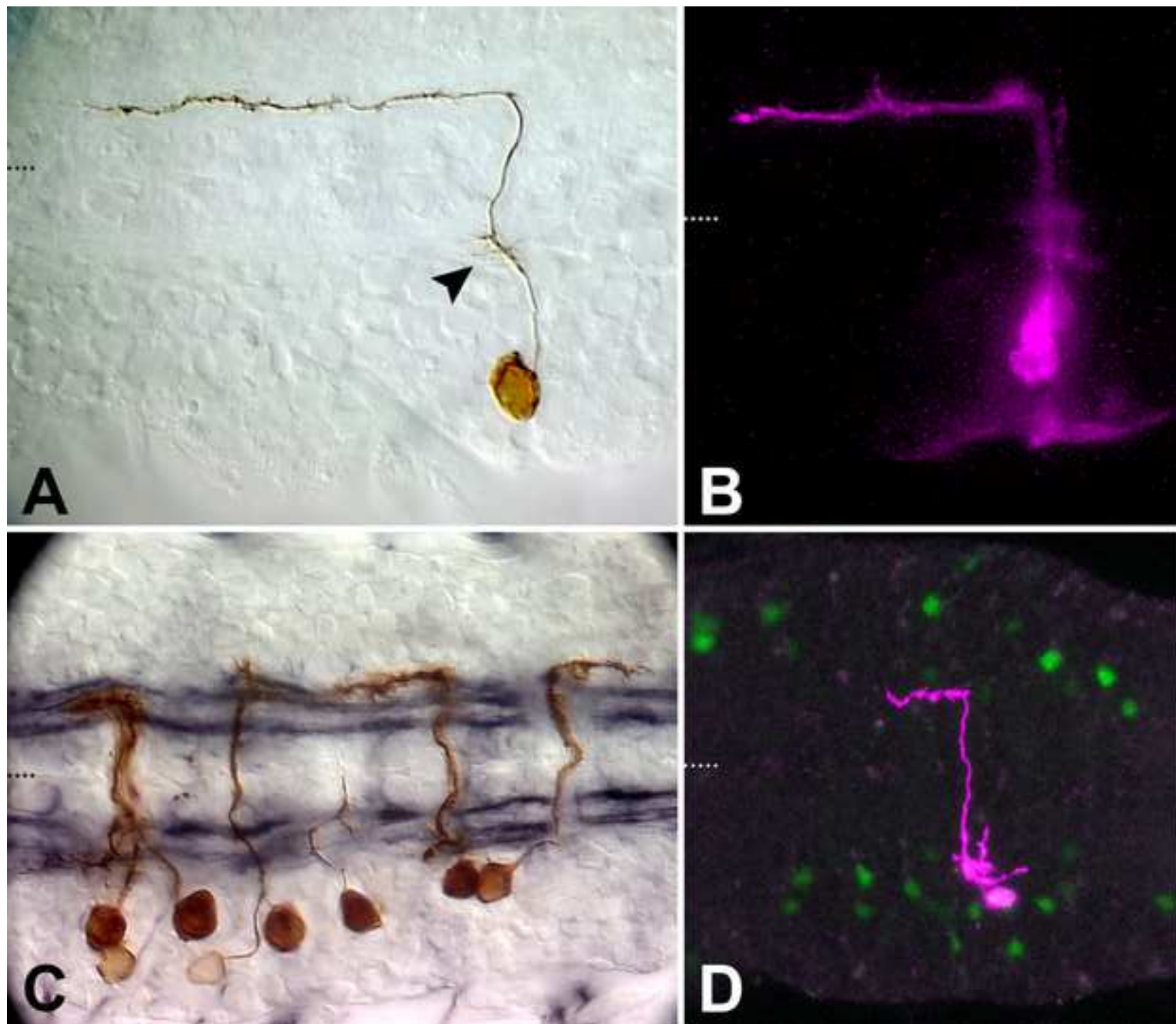
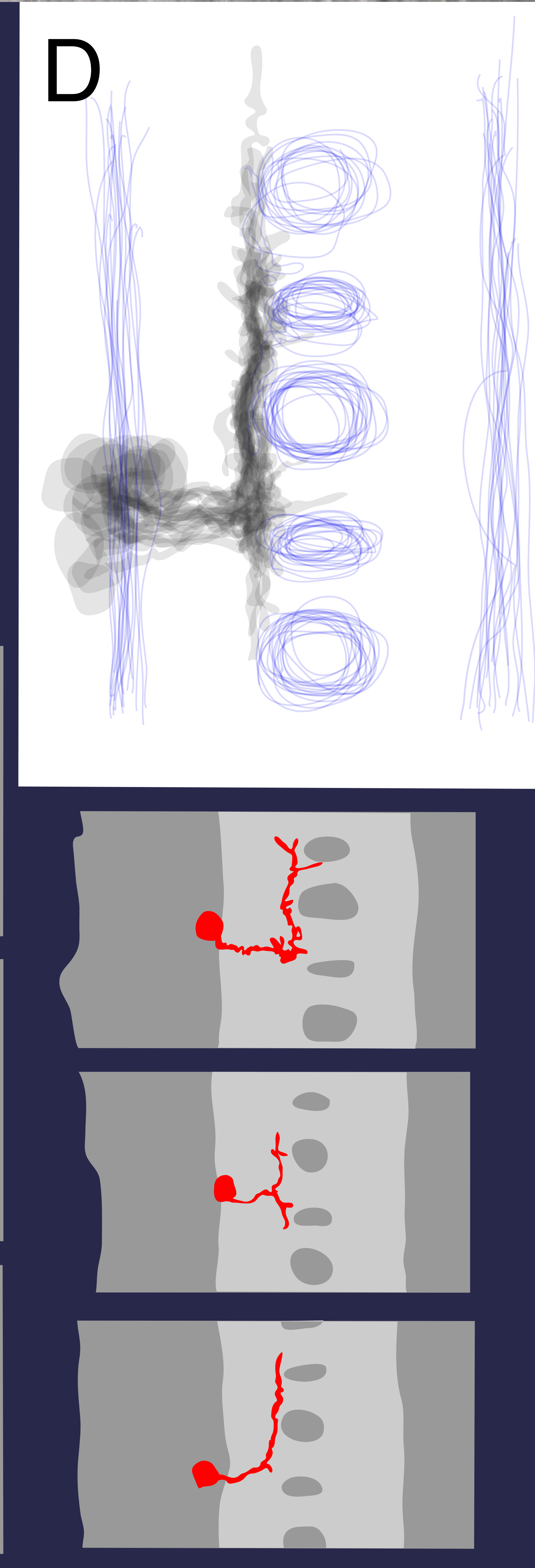
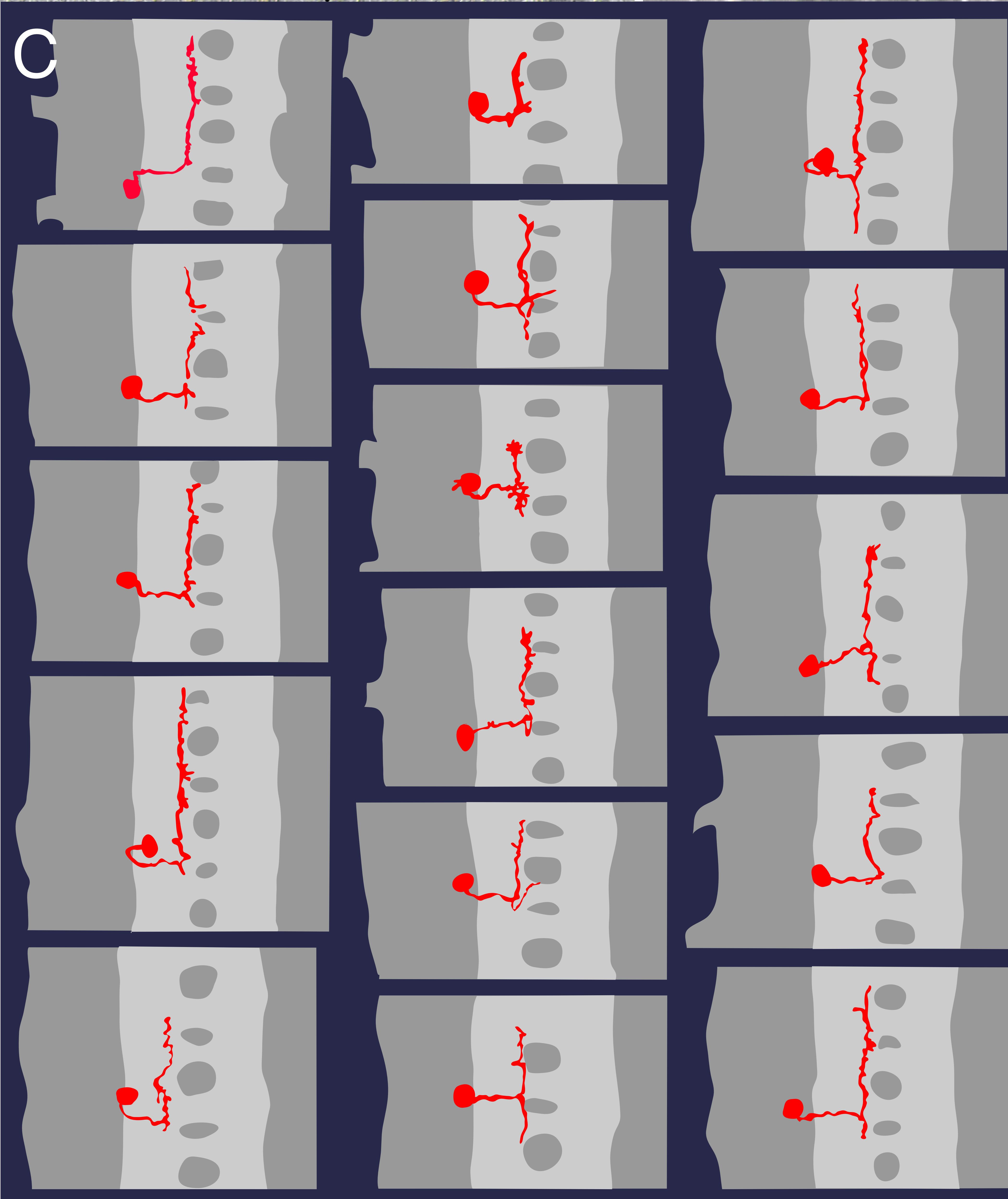
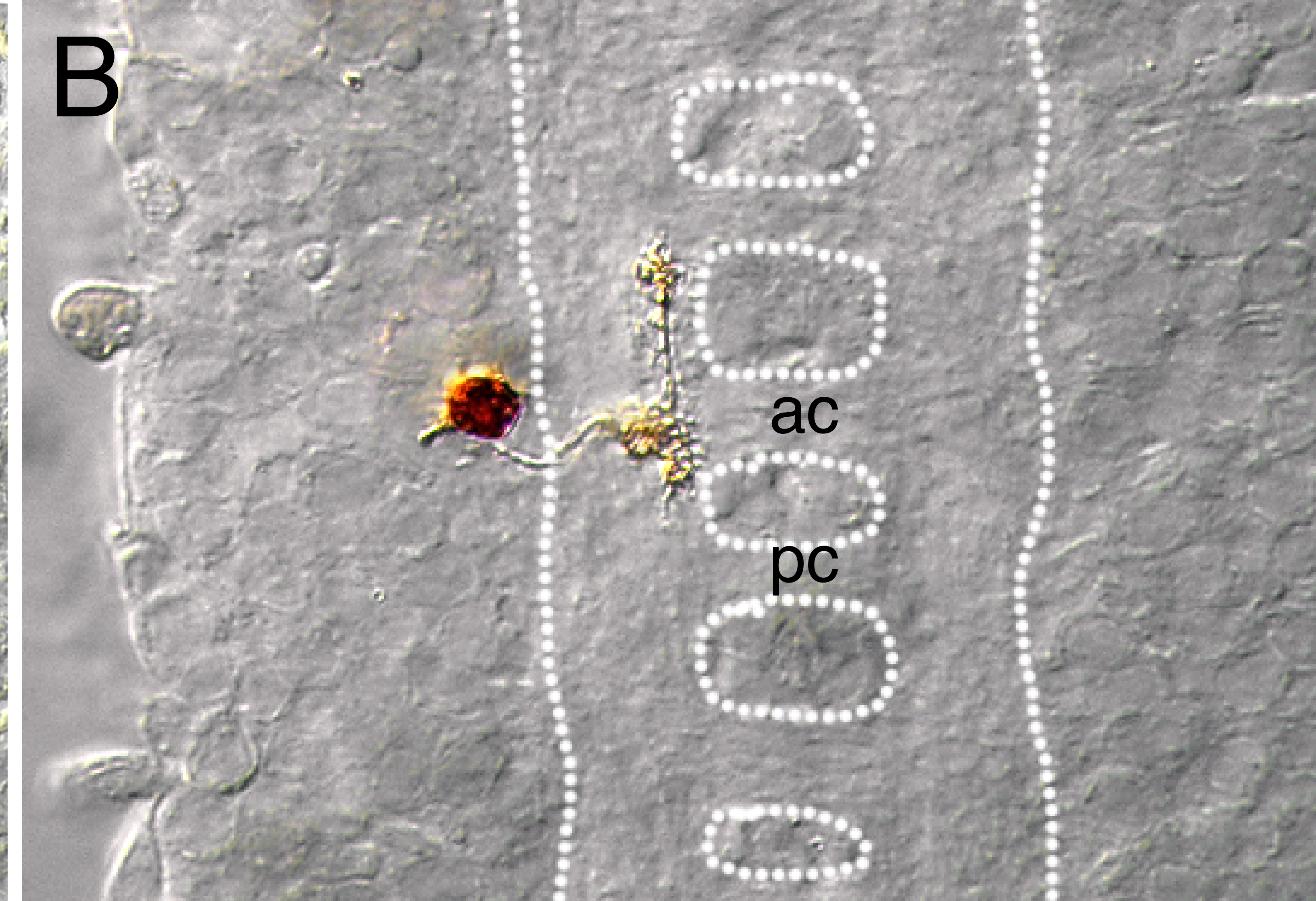
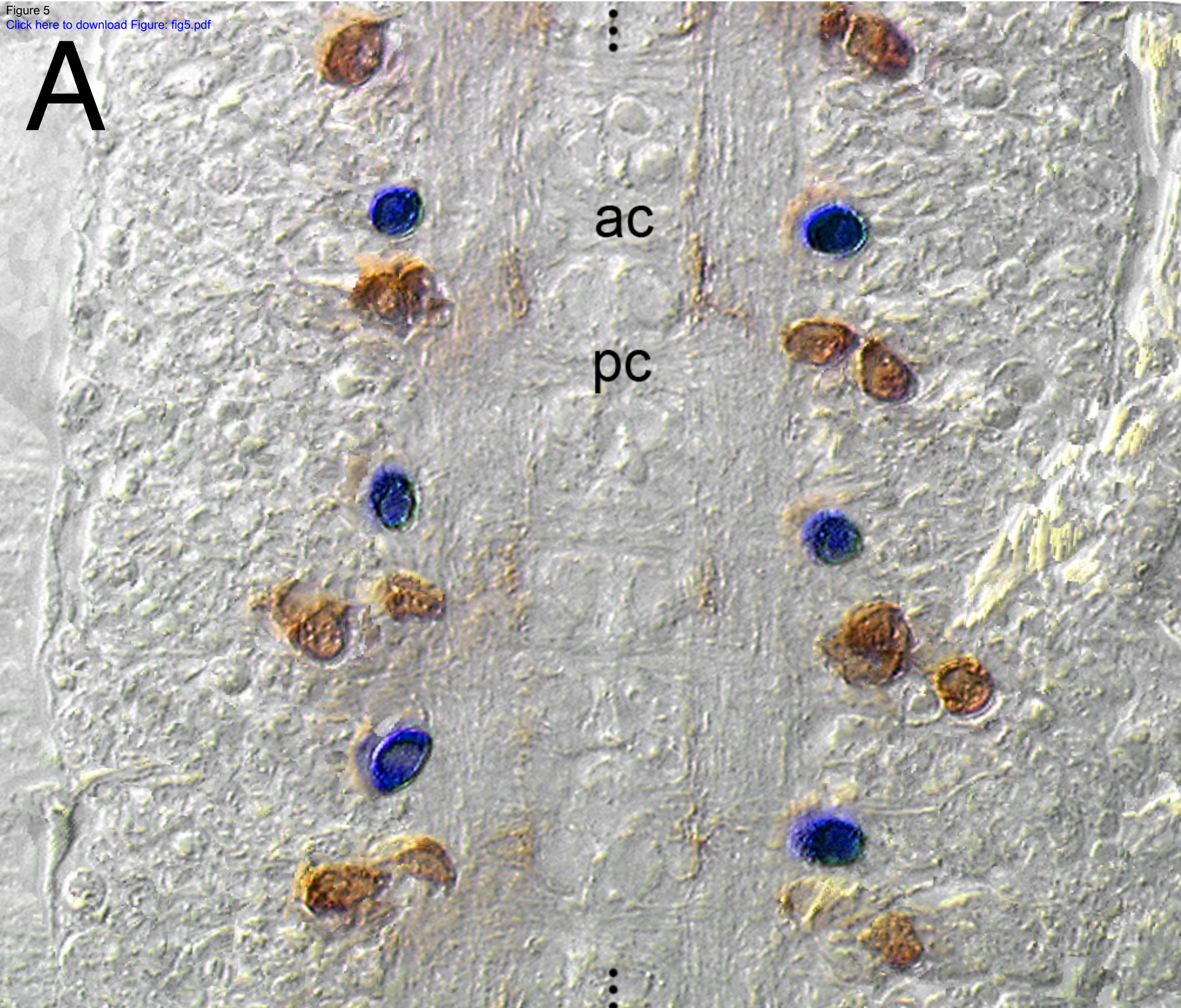


Figure 4
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Name of Reagent/Material	Company	Catalogue Number	Comments
REAGENTS			
DAB	Sigma-Aldrich	D-5905	2-3mg/ml in 100mM TRIS-Hcl, pH 7.4
Dil	Invitrogen/Molecular Probes	D-282	1 mg/ml in ethanol
Formaldehyde	Merck Millipore		7,4 % in PBS
Glycerol	Roth	3783	70% in PBS
Heptane Glue	Beiersdorf AG	Cello 31-39-30 *	dilute ca. 1 to 1 with n-Heptane
PBS			1x
TRIS	Roth	4855	
Vectashield	Vector Laboratories	H1000	
EQUIPMENT			
Confocal Microscope	Leica	TCS SP2 Cornerstone ION- 100	to view and document labelings in fluorescer to perform the labelings
DC - amplifier	Dragan Corporation	BB018018A1	18 x 18 mm
Coverslips	Menzel	BB024060A1	24 x 60 mm
Coverslips	Menzel		
Disecting Microscope	Leica	MZ8	to prepare and dissect embryos
Flat Capillaries	Hilgenberg		outer diameter 1mm; glas thickness 0.1mm
Injection Capillaries	Science Products	GB 100 TF 8P	
Micromanipulator R	Leica		to perform the labelings
Model P-97	Sutter Instruments		to pull capillaries
Object Slides	Marienfeld Superior	1000000	
Scientific Microscope	Zeiss	Axioscop 2 mot	to view labelimgss after photoconversion
Scientific Microscope	Olympus	BX50	to perform the labelings
Sony MC3255 Video Camera	Sony/AVT Horn	Sony MC3255	to record labelings after photoconversion

* This seems not to be an regular product. We phoned the company and explained what we need it for and they where happy to send it.

Weblink

<http://www.sigmaaldrich.com/catalog/product/sigma/d5905>

<http://products.invitrogen.com/ivgn/product/D282>

http://www.merckmillipore.com/germany/chemicals/formaldehydloesung-min-37-prozent/MDA_CHEM-104002/p_Wt6b.s1L6pAAAAEWpOE

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 Author(s): Christof Rickert, Thomas Kunz¹, Kerri-Lee Harris, Paul M. Whittington, Gerhard M. Technau¹

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Reply to Reviewers' comments:

Reviewer #1:

Summary:

The authors outline a technique that has been very influential in pushing forward our understanding of nervous system structure, organization and development, namely the manual labeling of neurons with Dil. They rightly assert the importance and continued usefulness of this method. The execution is clearly presented in a way that should allow novices to get set up and started. The supporting figures are absolutely wonderful - very clear and visually extremely pleasing.

Minor Concerns:

I would like the authors to consider the following, which I think might improve this manuscript further.

Page 6: 3) Filling of injection micropipettes: it might be helpful to point out that 'dry' absolute EtOH is needed, prepared by using 'molecular sieve' or similar for absorption of any residual humidity. 99.6% EtOH stocks can be sufficiently humid to cause Dil precipitation, which should be avoided at all cost.

Reply: a note has been added accordingly.

Page 6: Equipment: the objectives required are 'dipping' objectives, i.e. do not require a cover glass between lens and specimen, as 'immersion' objectives would.

Reply: We point out in the text: 'This objective should be designed for use without a coverslip'

On filter sets, it might help to provide notes on the excitation and emission maxima of Dil and a range of filter sets that would do the trick: e.g. sets suitable for Cy3 imaging, but also for Texas Red, Rhodamine or Alexa 568 / CF568 fluorophores (i.e. Dil has a pretty broad spectrum).

Reply: We added the maxima of Dil and several filter sets that will work beside the Cy3 set.

On the foot switch: perhaps to state that this can be helpful, but I doubt it is necessary. In my view, to facilitate use of this method, everything should be done to simplify as much as possible, especially on the equipment side, which will be pretty specific and expensive for most labs. In other words, it would help to clearly differentiate between the essential

items (and why these are essential), perhaps suggest cheaper alternatives, and the useful add-ons.

Reply: A sentence was added that makes clear that we performed the experiment also without electric shutter. Regarding the other items in this chapter we think they are all necessary.

Step 4.9: Add that after application of Dil specimens should be kept in a humidified chamber protected from light to avoid bleaching.

Reply: A corresponding statement was added.

On DAB conversion:

Here it would be good to point out possible pitfalls; e.g. is the quality of DAB important? Do specimens need pre-incubation with DAB prior to photoconversion? Will objectives be damaged by a DAB precipitate? What about toxicity of DAB and its disposal? How does fluorescence loss during the procedure relate to gain of DAB precipitate?

Reply: A new paragraph addressing these points has been added to the chapter on „Photoconversion“.

Table of reagents:

Most of the "labels" may need changing to "labeling" or similar - please check.

Reply: 'labels' were changed to 'labeling'

Reviewer #2:

Summary:

This is an important technique with excellent cellular resolution that has been used successfully over many years and continues to be important currently as shown by several excellent recent publications from the Technau lab.

Minor Concerns:

The authors might want to include a reference to the most recent paper from the Technau lab that uses this technique (i.e. Kunz et al., 2012).

Reply: We added the reference.

In part 6.1 a step 4.10 is mentioned, but this step does not exist.

Reply: 4.10 was corrected to be 4.9

Figures 5C and 5D are mixed up.

Reply: We uploaded the correct Figure.