Grundstudiumspraktikum Biologie II 2018 Praktikum in Zellbiologie: Teil Gewebe-Entwicklung

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Assistants:

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Organization:

1. The students will work in groups of 2 (exceptionally 3). Altogether there will be 22-25 groups, with 3-5 groups per assistant. There are altogether 7 assistants involved.
2. We have available 12 fluorescence microscopes equipped with a digital camera and computer. 2 groups (4 students) will share 1 microscope and work under the supervision of an assistant. Every assistant will supervise 2 to 3 microscopes. Every student will have the chance to perform immunohistochemistry, acquire and post-process images.
3. Every student will be individually evaluated based on the answers and tasks performed as instructed on the “Results and evaluation” section of the protocol (starts at page 14).
4. The signature of the assistant on the “Performance Sheet” is required for the certification.

NOTE: **No posting the results from this practical course anywhere**. We share with you some of our unpublished material which is currently strictly confidential.

Enjoy your course and learn a lot!

Jorge Pereira

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1. Immunofluorescence.

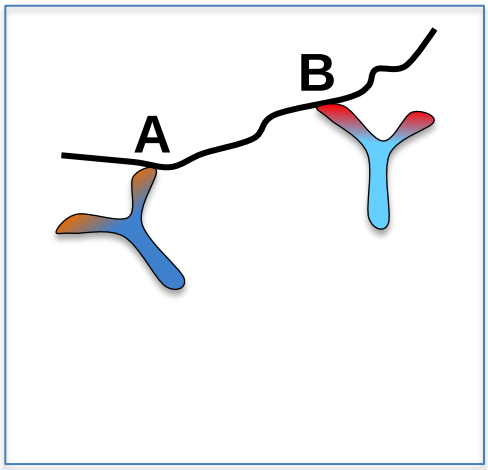
Immunohistochemistry consists essentially of applying an antibody recognizing a specific antigen to tissue sections, and then using some form of a label to detect those antibodies. Primary antibodies can be generated by exposing immune cells from animals of various species to the specific antigen which they recognize as foreign. These antibodies are purified so they can be used as a molecular reagent in a laboratory or applied in molecular medicine therapies.

We can classify immunofluorescence into 2 general types, direct and indirect.

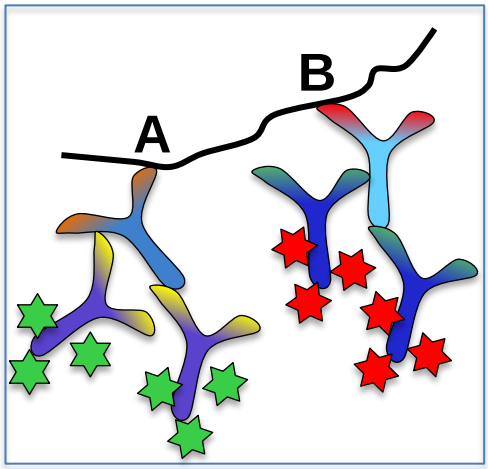
a) Direct immunofluorescence means that the primary antibody is directly coupled to a fluorophore. This means that the sample only needs to be exposed to a single antibody and is ready for detection. This is particularly useful if time is of concern in a particular experiment. For example, for labelling live cells that need to be FACS sorted and then used for further purposes you would like to have the labelling done as fast as possible to keep them viable, so using only one antibody will speed up the process.

b) Indirect immunofluorescence means that the primary antibody is not coupled to a fluorophore. Instead, there is a secondary antibody involved which recognizes IgG's of the animal species in which the primary antibody was generated. For example, if the primary antibody was generated in rabbits and targets the protein Ki-67, then the secondary antibody should be generated in a different species (goat or donkey for example) and it targets the IgGs of rabbits. The secondary antibodies can bind at multiple locations of the primary; therefore it is frequent that several secondary antibodies can bind a single primary simultaneously. Which are the advantages of this system? Please answer the questions in the **"Results and Evaluation"** section in page 14 of this protocol regarding the advantages you can think of.

Figure 1: Schematic representation of 2 primary antibodies (produced in 2 different species) targeting 2 different antigens, and respective secondary antibodies coupled to different fluorophores.



Different primary antibodies recognizing antigen A and B



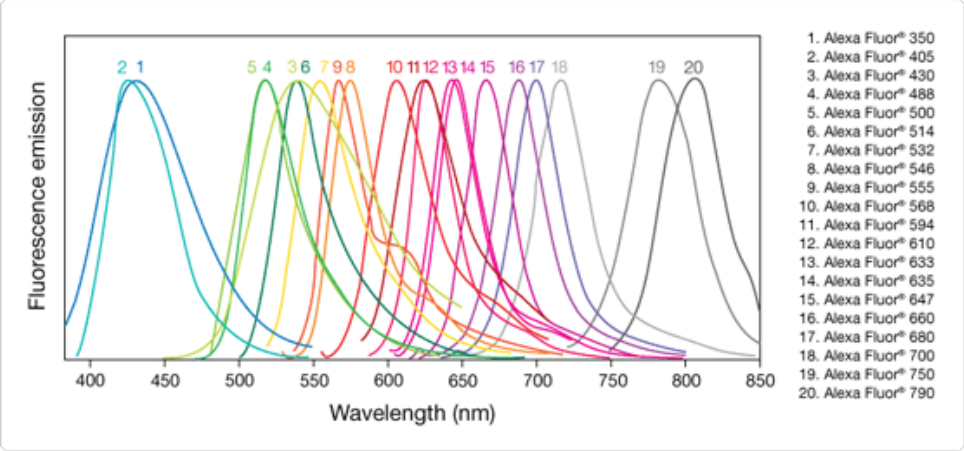
Secondary antibodies coupled to different fluorophores

In order to perform immunofluorescence to simultaneously detect multiple antigens there are a couple of aspects to consider:

a) The species in which the primary antibodies were produced should not be the same (when using the same immunoglobulin isotype)
b) The fluorophore coupled to the secondary antibodies recognizing the different species should also be different. Fig. 2 shows a few of the commercially available fluorophores which can be coupled to antibodies.

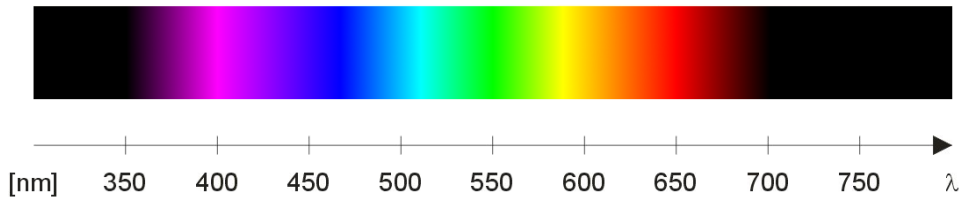
Please consider why is it important to comply with the 2 conditions stated above, and answer the questions in the **"Results and Evaluation"** section in page 14 of this protocol.

Figure 2: representation of the emission spectrum for a few common fluorophores, some of which are used in this practicum.



http://www.lifetechnologies.com/ch/en/home/references/molecular-probes-the-handbook/technical-notes-and-product-highlights/the-alexa-fluor-dye-series.html

Approximate visible Spectrum to the human eye



http://www.giangrandi.ch/optics/spectrum/spectrum.shtml

For further information on immunostaining and light microscopy see (i) Lodish, et al., "Molecular Cell Biology", Chapter 5.1, "Microscopy and Cell Architecture", especially the section "Fluorescence microscopy can localize and quantify specific molecules in cells" and/or (ii) Alberts, et al., "Molecular Biology of the Cell 5th edition", Chapter 9, "Visualizing Cells", especially the section "Antibodies can be used to detect specific molecules".

2. Axon-myelin interactions in the developing nervous system

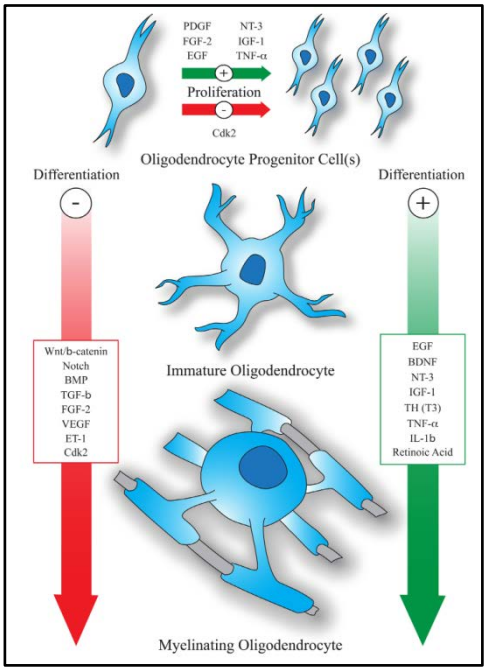
The development of the nervous system starts in early embryonic stages and continues until adulthood, both in mice and in humans. Furthermore, the nervous system displays a remarkable plasticity throughout the entire life, changing its structural and functional circuitry to accommodate continuous learning,

replenishing specific cell types as they perish, and activating repair mechanisms upon various lesion and disease scenarios. The nervous system is composed of 2 major compartments, one of which is the central nervous system (CNS), which includes the brain, spinal cord and optic nerve. The CNS is itself connected to the majority of the organism through the peripheral nervous system (PNS). The central and peripheral nervous system does not only consist of neurons and axons, but also contains numerous other cell types. Orchestrating the proper generation of these cells and their correct localization in the nervous system requires a coordinated signaling cascade, and is important to form a properly functioning nervous system. These cells carry out highly specialized and important roles, and a prime example of such specialization is embodied by the myelinating cells. Myelination is carried out by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. Myelin consists of a lot of cell membrane concentrically wrapped and compacted around the axons to provide electrical insulation and allow the propagation of nerve signals quickly and without fading throughout the axon. The myelinated segments in the axons, termed internodes, are not continuous, and in-between each myelinated segment there is a highly specialized region termed “Node of Ranvier”. The region around the Node of Ranvier is tightly organized and contains clusters of voltage-gated sodium and potassium channels. This organization enables the reinforcement of the action potential so it can travel successfully across the following myelinated segment until it reaches its target. Even though myelination serves similar purposes in the CNS and PNS, there are some fundamental differences in the myelin composition and in the behavior of the myelinating cells in the 2 compartments. For example, some of the proteins incorporated into the myelin sheet are only present on Schwann cells, and others only on oligodendrocytes. In your immunofluorescence you will detect one myelin protein, P0, which is exclusively expressed by either Schwann cells or oligodendrocytes. Which one? Look carefully at the results you obtain in the practicum and answer the questions in the **“Results and Evaluation”** section in page 14 of this protocol. But there are other notable differences between the 2 cell types. A Schwann cell can only myelinate a single axon segment, whereas an oligodendrocyte can engage and myelinate many (see Fig. 3). Schwann cells are very plastic cells which can reverse their differentiation state when there is a nerve injury in the PNS (Fig. 3, red arrow from Myelinating Schwan cell to repair cell). This feature is crucial for nerve repair, since after de-differentiation Schwann cells are able to secrete factors that promote survival and regrowth of injured axons in the PNS. This Schwann cell ability, coupled to efficient clearing of myelin debris (which includes the recruitment of immune cells), is thought to underlie the robust regeneration observed in the PNS upon injury. Oligodendrocytes are thought to be less plastic and to have a limited ability to shift their differentiation state. It is also well documented that the CNS is less able to regenerate efficiently upon injury, and a part of this limitation comes from inefficient clearing of oligodendrocyte myelin debris in the lesion area. The maintenance of the myelin sheet is also very important to preserve the health on a non-injured nervous system. Demyelinating neuropathies, such as multiple sclerosis in the CNS and Charcot-Marie-Tooth (CMT) disease in the PNS, have a strong detrimental impact on the lives of affected patients, many of which are young adults which find themselves disabled. It is therefore of crucial importance that we deepen our understanding of how both myelinating cells differentiate and of how this process is regulated at the molecular level, so this knowledge can eventually be transplanted into the medical field to aid the design of new therapeutic

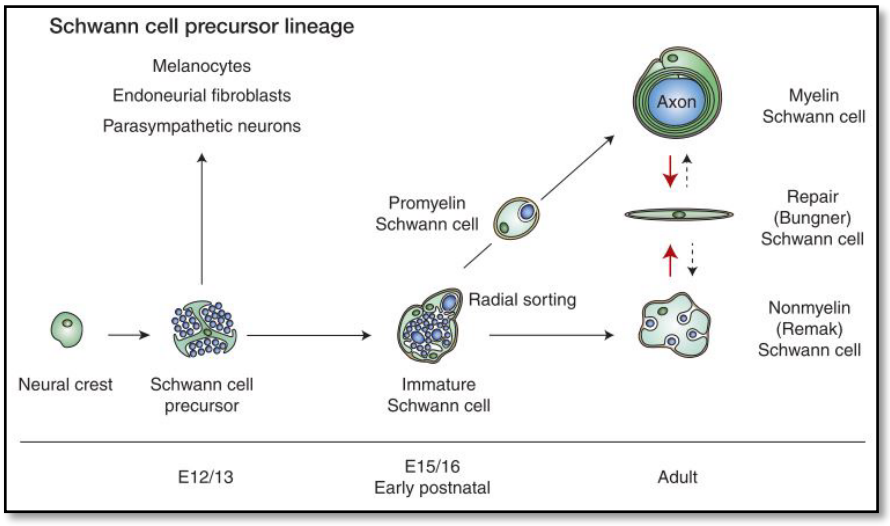
approaches to traumatic lesions and demyelinating neuropathies, among other diseases which involve the myelinating cells.

Fig.3 – Oligodendrocyte and Schwann cell development.

a) Oligodendrocyte development
Gallo et al, 2014



b) Schwann cell development
Jessen et al, 2015



If you wish to learn more about myelinating glia, please read:

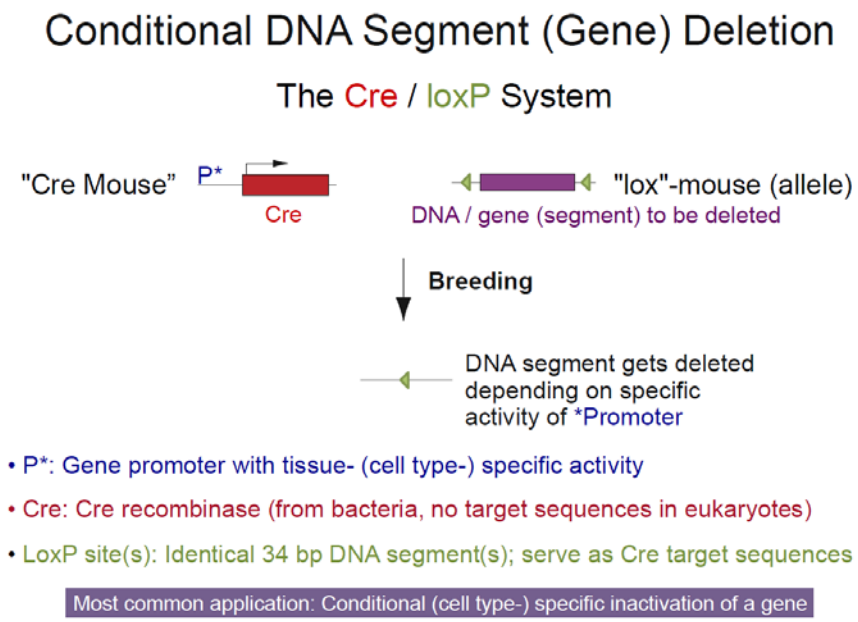
1. Two reviews about the CNS glia development:
    a. Gallo, V., & Deneen, B. (2014). Glial Development: The Crossroads of Regeneration and Repair in the CNS. Neuron, 83(2), 283–308. doi:10.1016/j.neuron.2014.06.010
    b. Chang, K. J., S. A. Redmond and J. R. Chan (2016). "Remodeling myelination: implications for mechanisms of neural plasticity." Nat Neurosci 19(2): 190-197.
2. Two reviews about Schwann cell development:
    a. Jessen, K. R., R. Mirsky and A. C. Lloyd (2015). "Schwann Cells: Development and Role in Nerve Repair." Cold Spring Harb Perspect Biol 7(7): a020487.
    b. Taveggia, C. (2016). "Schwann cells-axon interaction in myelination." Curr Opin Neurobiol 39: 24-29.

3. The use of conditional mutant animals as a valuable genetic tool for research

The mammalian nervous system is a complex system, and myelination is a prime example of axon-glia interaction that requires the presence of myelinating cells, axons, and the physiological context of the nervous system itself. Due to its complexity, there is currently no prime alternative to investigate the relation between these cells in detail other than to use animal models of research, most notably the mouse model. A modern and very precise way to evaluate the importance of a specific protein in a specific cell type is to generate a conditional mouse mutant. A widespread and robust approach to generate conditional mouse mutants is the

Cre / LoxP system. LoxP sequences are 34bp long DNA sequences which do not normally exist in the mammalian genome. The cre recombinase (Cre) is an enzyme that specifically recognizes 2 nearby LoxP sequences in the genome, binds both sequences and cuts the genomic region in-between, leading to a *de facto* deletion of this cleaved DNA portion. By placing LoxP sequences flanking both sides of a crucial exon for the protein you want to investigate, you generate a floxed mouse for that protein. By breeding this animal with another animal that expresses Cre, you can obtain a mutant with a deletion for the protein you are investigating (Fig. 4).

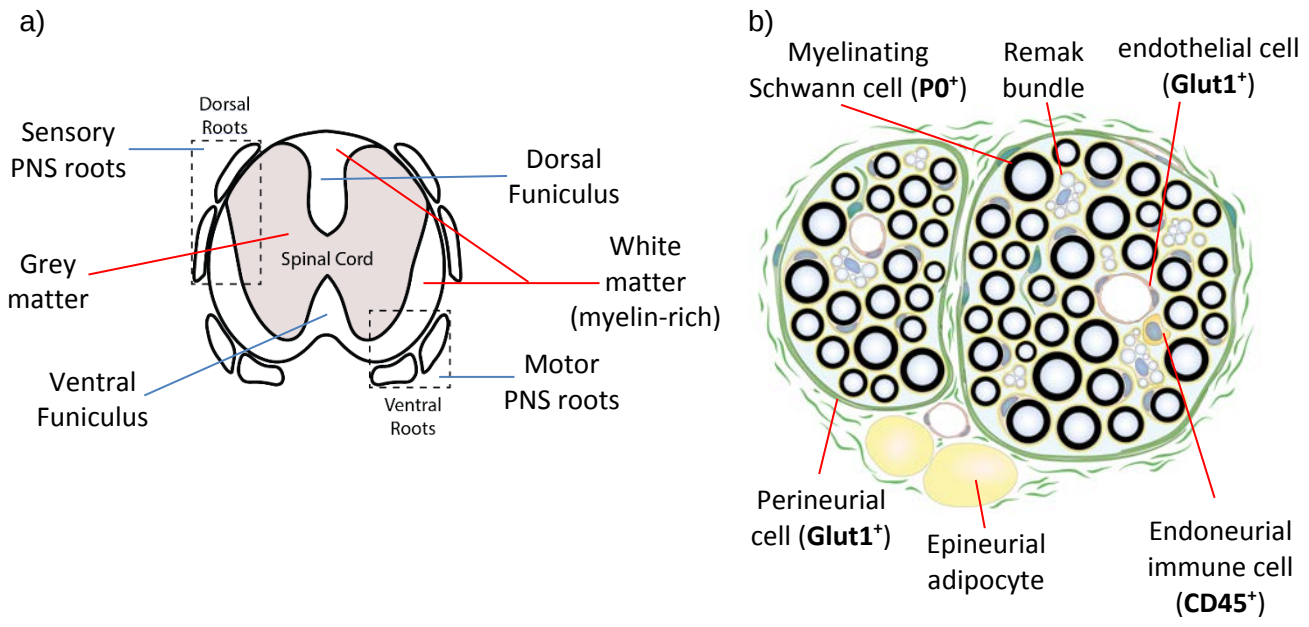
Figure 4 – Schematic representation of the Cre / LoxP system (taken from Prof. Suter concept course lectures in cell biology, 2016)



Deleting an important protein from every cell in the organism is frequently embryonic lethal, and you may not learn as much about the importance of that protein in each specific cell type. The animals would show a sum of phenotypes originating from various cells. To ensure that the protein is only deleted in a single cell type, the Cre expression must be regulated by a promoter that is only active on that specific cell, at least in the organ you are analyzing. Returning to myelination as an example, by comparing such mutant animals with wildtype littermates it is possible to address the importance of a specific protein for myelination by oligodendrocytes or by Schwann cells. Mutant animals may develop phenotypes or defects in myelination not present in control animals. If you look at Figure 3 from this protocol you can see several factors that are known to promote or suppress myelination in the CNS. Numerous homologous studies have also been performed in the PNS. Much of this knowledge was gained through the analysis of conditional mutant animals for individual proteins. In this practicum the work is performed in groups of 2 people. One receives a slide containing sections of spinal cord (CNS) with adjacent roots (PNS) (See Figure 5a) for a control and a conditional mutant animal, in which Dnm2 has been deleted from Schwann cells. Is the CNS or the PNS affected by the mutation? Look carefully at the results you obtain in the practicum and answer the questions in the **“Results and Evaluation”** section in page 14 of this protocol. The other group member receives a

slide containing sciatic nerve sections also derived from Dnm2 control and deletion-mutant animals (see Fig. 5b). Deletion of Dnm2 may a) promote myelination, b) inhibit myelination or c) not affect myelination to an extent that can be evaluated through immunofluorescence. It is up to you to determine the changes that happen in the mutant tissue (per slide). Look carefully at the results you obtain in the practicum and answer the questions in the **"Results and Evaluation"** section in page 14 of this protocol. Besides of the immunofluorescence, you will receive an introduction to transmission light microscopy as well. In this introduction you will be shown sections from resin-embedded tissue blocks termed semithin sections. These are 0,5 μm thick, and are much better morphologically preserved than the 10 μm frozen sections you use in immunofluorescence. Unfortunately, the processing that enables such a strong morphological preservation makes the antigens inaccessible for antibodies and unsuitable for immunofluorescence. In these slides, you will be able to compare 4 different mutants that carry a specific deletion of 4 different proteins in Schwann cells and/or oligodendrocytes. The PNS tissue sectioned is the sciatic nerve, a large peripheral nerve in the leg frequently used for research in the PNS. The CNS tissue consists of the spinal cord, with the surrounding PNS roots also visible. Look closely at those sections, shoot some pictures and discuss your impressions with the assistants. Just like for the immunofluorescence, you will have to judge whether these proteins most likely promote myelination, inhibit myelination or do not affect myelination to an extent that can be evaluated with semithin sections. There are also a few questions for you to answer in the **"Results and Evaluation"** section in page 14 of this protocol. Besides of its use in the generation of deletion mutants, the Cre / LoxP system can also be used to induce expression of specific (even foreign) proteins in a cell-specific manner, including reporter proteins that allow researchers to follow the fate of specific cell types in an organism. For further information regarding the Cre / LoxP system please visit http://cre.jax.org/introduction.html

Figure 5 – a) Schematic layout of the adult Spinal cord (CNS) with surrounding nerve roots (PNS) in the lumbar region; b) Schematic representation of a peripheral nerve, such as the sciatic nerve



Adapted from Deniz Gökbuget

Adapted from Daniel Gerber

Goals of the practical course

1 To introduce you to the analysis of conditional mutant animals in which a particular protein is ablated in a specific cell-type. These genetic tools allow you to evaluate the consequences of this deletion and infer the physiological importance of this protein in the cell-type you are investigating. With this method we have gained valuable knowledge that deepens our understanding of cellular and molecular processes in healthy scenarios and also in diseases which are relevant for humans.

2 To show you the product of the nervous system development, and to highlight the presence of different myelinating cells engaging the axons in the central and peripheral nervous system. Even though the electrical insulation purpose of the myelin sheet is shared between the CNS and the PNS, the cells that perform this task, the composition of the myelin they produce and the additional supportive functions they serve are substantially different between the 2 compartments. These cells also play different roles in nerve regeneration upon injury or disease, and therefore are important subjects of the research we perform in our laboratory. Beyond the myelinating cells, we will look at some of the cells that compose peripheral nerves as an organ, and how these cells should be localized after development.

3 To tutor you on how to assemble a data panel with immunofluorescence pictures. There are ground rules that you should follow in order to accurately represent your data and efficiently convey your message.

4 A practical goal is to provide a hands-on introduction to immunofluorescence, a basic fundamental technique used in numerous research projects. Every student should have the opportunity to perform the immunofluorescence protocol, take pictures and gain experience with microscopy and computer-aided image processing.

Protocol — Read before you start your experiments!

Focus in your tasks and be careful. This is a sensitive experiment that will not work if you make mistakes.

1. Sectioning nervous system samples (done by assistants)

Due to limited time, an assistant has performed part 1. The experimental steps are listed below for your information.

1. Neural tissue was collected from 24 day old control and mutant animals, after intra-cardiac perfusion with 4% *para*-formaldehyde (PFA).
    a. One sample consists of the spinal cord (CNS) with the attached nerve roots (PNS).
    b. Another sample consists of the sciatic nerve, a major peripheral nerve.
2. The tissues were further fixed in 4% PFA for 1.5 h at room temperature.
3. The tissues were incubated for 1h in 10% sucrose/PBS and overnight in 20% sucrose/PBS.
4. The samples were embedded in Tissue-Tek OCT (thick liquid at room temperature, solid at -20).
5. 10 µm thick frozen sections were cut in a cryostat.
    a. The samples containing spinal cords and adjacent nerve roots sections were sectioned at the medial lumbar area. The PNS roots in this area are large and are aligned parallel to the spinal cord, which makes it convenient for our analysis.
    b. Sections of the sciatic nerve were harvested on a separate microscopy slide.

2. Immunohistochemistry

2.1. Material:

1. Mixture of primary antibodies (aliquots prepared by the assistants):
    a. 1 tube with a mixture to add to spinal cord and adjacent roots slide containing rat anti-MBP (diluted 1:125 in blocking buffer), mouse anti-Neurofilament and chicken anti-P0 antibodies (both diluted 1:250 in in blocking buffer);
    b. A separate tube with a mixture to add to the sciatic nerve slide containing rat anti-CD45, rabbit anti-Glut1 and chicken anti-P0 antibodies (all diluted 1:250 in blocking buffer)
2. Mixture of secondary antibodies to perform immunolabelling of the primary antibodies bound to the neural tissue sections.
    a. One tube with a mixture to add to spinal cord and adjacent roots slide containing goat anti-rat IgG, goat anti-mouse IgG, and donkey anti-chicken IgG, each antibody type coupled to a different fluorophore
    b. Another tube with a mixture to add to the sciatic nerve slide containing goat anti-rat IgG (to detect CD45), goat anti-rabbit IgG (to detect Glut1) and donkey anti-chicken IgG (to detect P0), each antibody type coupled to a different fluorophore

c. *Figuring out which fluorophore is coupled to each antibody can be deducted by interpreting the images you acquire at the microscope*, and you have to fill in this information in the "**Results and evaluation" section of this protocol starting on page 14**. Secondary antibody aliquots were prepared by the assistants and are diluted 1:250 in blocking buffer.

3. Blocking buffer. Blocking buffer is 10% goat serum, 0,1% Triton-x-100 detergent in phosphate-buffered saline (1X PBS). Blocks non-specific binding of antibodies.
4. 1 x PBS
5. A wet chamber
6. 1 slide with Spinal Cord (CNS) and adjacent root (PNS) sections harvested from control and Schwann cell specific mutant animals (slide with orange dot).
7. 1 slide with sciatic nerve sections harvested from control and Schwann cell specific mutant animals (slide with green dot).
8. Slide-washing plastic "mailers" (3 per person) with a rack
9. 1ml precision pipet
10. 1ml pipet discardable tips
11. 50x24 mm glass coverslips
12. Glass waste container
13. Timer
14. Pencil
15. "Immumount" mounting media.
16. Paper tissues
17. Squeeze bottle with deionized water

2.2. Method:

To prepare the wet chamber, add two Paper tissues papers on the bottom of the wet chamber box, and wet them thoroughly with water from the squeeze bottle. This is done to avoid evaporation of the antibody solution during staining.

1. Just before the practicum an assistant will incubate the slides for 10 minutes in pre-chilled methanol (-20°C) to permeabilize the tissue and facilitate the access of the antibodies to the antigens. The assistant then transfers the slides to PBS and brings them to the practicum laboratory.

2. **Each group of 2 people should have 2 slides**, one with an orange dot (labelled SC/Roots) and one with a green dot (labelled SN). If the dot comes off, read the labelling. Take the slides from the PBS and label them with pencil with your group name. Be careful never to touch the sections throughout the whole protocol.

3. *Steps 4, 6 and 9 are performed by placing slides horizontally in the wet chamber and adding the solution carefully onto the individual slides*. *Washing steps (8 and 10) in plastic slide mailers.*

Blocking

4. Add ~ 250 µl of blocking buffer to the slide.
5. Apply a coverslip to the slide: Start with the coverslip at an angle and touching one end of the slide, then very slowly lower the coverslip (using either your fingers or the forceps in your bench) to cover the slide and allow the liquid to smoothly advance and expel the air towards the other end. This method avoids trapping air bubbles. Close the lid of the wet chamber and incubate at room temperature (RT) for 30 min (in the wet-chamber). Read the protocol thoroughly in these 30 minutes and ask questions to your assistants.

6. After the incubation, drain off the blocking buffer.
    a. To remove the coverslip, either:
        i. tilt the slide vertical in a sudden movement and allow the glass to drop out into the glass waste container.
        ii. If the coverslip gets stuck to the slide, immerse both into PBS inside your plastic mailer and the coverslip will simply fall off. Grab the coverslip with your forceps and put it on the glass waste container in your bench. Do not force-remove the coverslip if it attaches to your slide, you will damage the tissue sections.

Primary antibody – During the 1h you will have an introduction to the microscopes, see page 13 of the protocol

7. Carefully add the aliquot (250 µl) of the **primary antibodies** to the slides lying in the wet chamber.
    a. Aliquot with the **orange dot and a "1"** is added to the SC/Roots slide also marked with an orange dot.
    b. Aliquot with a **green dot and a "1"** is added to the SN slide, also marked with a green dot.

8. Apply a coverslip on the slide (as explained in point 5). Close the lid of the wet chamber, and incubate at RT for 1h.

9. After the incubation, remove the coverslip (explained in point 6) and wash slides 3x, 5 minutes each time, in PBS at RT.

Secondary antibody – During the 1h incubation you will have your lunch break

10. Carefully add the aliquot (250 µl) of the **secondary antibodies** to the slides lying in the wet chamber. All fluorescent dyes – even the Alexa dyes- are light sensitive — *keep in the dark*. Don't forget to apply a coverslip as explained in step 5.
    a. Aliquot with the **orange dot and a "2"** is added to the SC/Roots slide also marked with an orange dot.
    b. Aliquot with a **green dot and a "2"** is added to the SN slide, also marked with a green dot.

    c. *Have your lunch break during this hour, but be back in time to continue the protocol*

After Lunch

11. Wash slides 3x, 5 minutes each time, with PBS
    i. Remove the coverslip as explained in step 6

12. Drain excess liquid with paper tissues, but be careful not to touch the sections. Put 2 drops of Immumount on the slide. Apply a coverslip as explained in step 5, and let dry (the Immumount mounting medium dries in about 10-15 min – until then, be careful not to move the coverslip.

After slides are dry, they are ready to be imaged with the microscope.

Note: take care that no Immumount sticks to be bottom of the slides, or it will dirty the microscope stage. Wipe the bottom (NOT the top) of the slide with a wet tissue before placing it on the microscope for the first time.

3. Introduction to the microscopes, practice fluorescence and transmission image acquisition

Please read the section "Microscopy and image processing" starting on page 19 for instructions of how to handle the microscope software before you look at the samples you labelled for immunohistochemistry.

The immunostaining involves some waiting time. During the primary antibody incubation you will use the 1h to acquaint yourself with the microscopes and the computer system, and take a first look at tissue sections using fluorescently labelled sections (only with DAPI). You also have the opportunity to look in greater detail at myelinated neural tissue using toluidine-blue stained semithin sections with brightfield transmission light microscopy. For these examples we selected myelinated samples of CNS and PNS from 6 different animals.

A) One control and three harboring mutations that eventually result in different myelin abnormalities in the PNS.
B) One control and one harboring a mutation that results in neuro-glial abnormalities in the CNS.

Look carefully and try to figure out what type of myelin aberration these animals present. Discuss with your assistants and colleagues.

1. Each microscope has 6 mounted slides (one stained with DAPI and 5 with semithin sections) available for demonstration and for you to look at. Please follow the introduction that the assistants will provide you.

2. After the introduction, sit at the microscope and browse through the samples, take a picture after you set a good exposure. At least 2 groups (4 students) will share 1 microscope throughout the practical course.
    a. You should look at least 1 of the semithin sections with transmission (not fluorescence) illumination. Shoot a picture with an appropriate exposure. Make sure to observe and discuss the semithins on the 5 other slides which your colleagues are browsing through on the same microscope. This is necessary for you to answer some of the questions. **Please see the Section 1 of the "Results and evaluation" section on page 14 and answer the questions.**

    b. Using the fluorescence illumination (make sure the transmission light is off), look at the slides stained with DAPI. Shoot a picture at an appropriate exposure. **Please see the Section 2 of the "Results and evaluation" sections and answer the questions.**

Results & Evaluation

Answer the questions in this section during the course of the day (ask the assistants for help if you have trouble figuring out the answer). When you are finished, present your answers together with the printed microscopy images to the assistant for evaluation. You will need to show them at the end to obtain your certificate.

1. Conceptual analysis of semithin sections:

a. Sciatic nerves (PNS) harvested from 1 control and 3 distinct conditional mutant animals.

b. Spinal cord (CNS) harvested from 1 control and 1 conditional mutant animal

1.1 *Look at the semithin sections (slides next to each microscope) using the 40X objective. Each student (out of the 4) will look at least at one semithin slide, but project the image on the camera/screen and shoot a picture so you* can discuss with each other (and the assistant) and answer the questions below.

1.2 The assistant examines one good semithin in the slide labelled “control” and shoots at least one picture at an appropriate exposure.

1.3 Can you identify the myelinated axons? (Look for the bright circles surrounded by dark myelin rings)

1.4 Student #1 examines the semithin sections in the slide labelled “TSC1 mutant” and shoots at least one picture at an appropriate exposure. Look at the monitor and compare the picture he/she acquired to the control picture.

1.5 Do you think the myelin is thinner, thicker or unaltered compared to control? A:_____

1.6 Student #2 examines the semithin sections in the slide labelled “Raptor mutant” and shoots at least one picture at an appropriate exposure. Look at the monitor and compare the picture he/she acquired to the control picture.

1.7 Do you think the myelin is thinner, thicker or unaltered compared to control? A:_____

1.8 Student #3 examines the semithin sections in the slide labelled “Dnm2 mutant” and shoots at least one picture at an appropriate exposure. Look at the monitor and compare the picture he/she acquired to the control picture.

1.9 Do you think myelin is more, less or equally abundant compared to control? A:_____

1.10 Do you notice any other changes in the Dnm2 mutant nerves? How does the nerve size compare to controls? A:_____; Are there changes on the cell types populating the endoneurium? A:_____? Suggest a hypothesis of what these abundant cells could be (see schematic on page 7):_____
_____. Discuss with your assistants.

1.11 Student #4 examines the semithin sections in the slides labelled “TDP43 control and mutant”, and shoots at least one picture at an appropriate exposure for both control and mutant samples. Look at the monitor and compare the mutant to the control picture.

1.12 Is the CNS or the PNS apparently affected in these sections? A: **CNS** or **PNS**

1.13 Do you think myelin is more, less or equally abundant compared to control? A:_____

1.14 Do you notice anything strange with the axons in this sample? A: **Yes** or **No**

1.15 If you answered yes in the question before, how to explain that the axons are affected in a glia-specific mutant? Does that allow you to propose a hypothesis regarding glial support towards axons? Hypothesis:_____
_____. Discuss with your assistants.

1.16 Based on your observations, which general role could be attributed to each of these proteins in your opinion? Circle the correct answer below:

a) TSC1 protein **promotes / inhibits / has no obvious influence on** myelination by **Schwann cells / oligodendrocytes**

b) Raptor protein **promotes / inhibits / has no obvious influence on** myelination by **Schwann cells / oligodendrocytes**

c) Dnm2 protein **promotes / inhibits / has no obvious influence on** myelination onset by **Schwann cells / oligodendrocytes**. Deletion of this protein **seems to affect** / **does not seem to affect** the cellular composition of the nerve.

d) TDP43 protein **promotes / inhibits / has no obvious influence on** myelination onset by **Schwann cells / oligodendrocytes**. Deletion of this protein also **seems to affect** / **does not seem to affect** the integrity of the axons.

Note: You do not need to keep or print these pictures.

2. Introduction to fluorescent microscopy using DAPI-stained sections

2.1 Examine the slide stained with DAPI *(slide next to each microscope)* using the blue light filter. Get familiar with fluorescence microscopy and try to identify the spinal cord and adjacent nerve roots. Practice how to handle the reflective light shutter, how to change filter and adjust exposure times.

2.2 Take a picture at a suitable exposure.

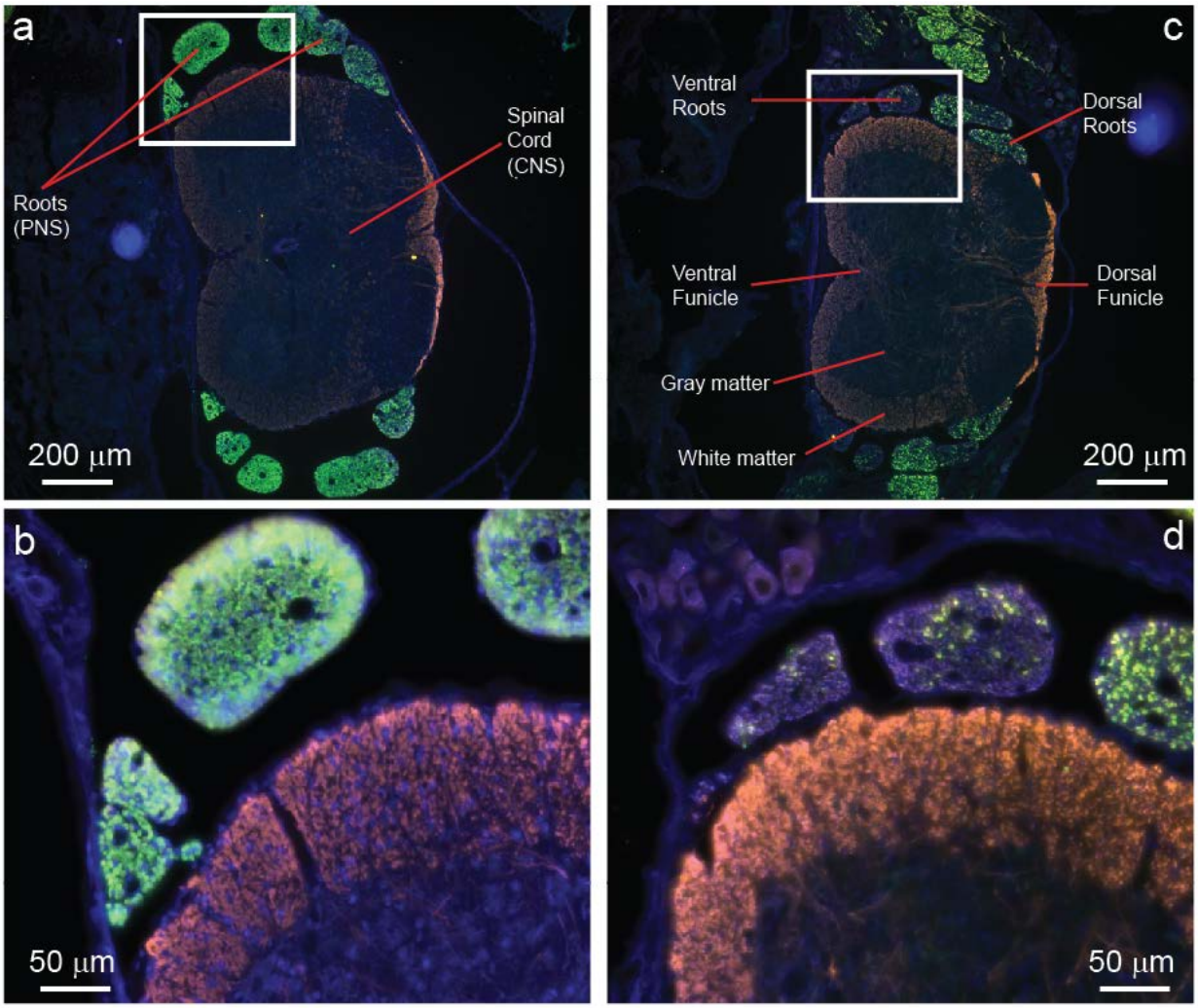
2.3 What cellular structure do you see labelled with DAPI? _____

2.4 Do you think DAPI interacts strongly with RNA? **Yes** or **No**. Why?_____

Note: You do not need to keep or print this picture.

3 Your Immunofluorescence:

Each of you must take pictures of the slide you worked with. The position of the control and mutant sections on the slide is explained in the laminated panel attached to each microscope. Please follow the "microscopy guide" starting on **page 19** of this protocol. Take your pictures with the 40x (recommended) objective in the area around the ventral roots, capturing part of the roots (PNS) and of the spinal cord ventrolateral white matter (CNS) – region marked by white box in the images below.



3.1 Slides with Orange dot – Spinal cord and adjacent roots from control and mutant animals

3.1.1 Shoot the pictures of the slides you worked with (control and mutant sections) using 3 filter sets (when you look through the microscope oculars the fluorescence of each filter looks blue, green and red). – DO NOT MOVE THE SLIDE until all 3 colors are photographed in each section.

3.1.2 Can you identify the Spinal cord (CNS)? And the nerve roots (PNS)? Be aware that some of the roots may lie very close to the spinal cord

3.1.3 Can you match the color channels to the labelled antigen? Circle the correct answer below (Label the proteins detected in each color in your pictures on the computer before you print them)

Blue: **Neurofilament / P0 / MBP**

Green: **Neurofilament / P0 / MBP**

Red: **Neurofilament / P0 / MBP** as

3.1.4 Is P0 expressed in the CNS? A:_____

3.1.5 Based on your observations, is this mutation affecting the CNS (spinal cord) or the PNS roots? A:_____

3.1.6 Are P0 and/or MBP expressed in the axons? A:_____

3.1.7 Which *fluorophore* was most likely coupled to each secondary antibody added to SC/Root sections? See the spectrum Fig. 2 on page 3 and the primary antibody species to answer.

Goat anti mouse: Alexa 405 / Alexa 488 / Alexa 546 / Alexa 750

Goat anti chicken: Alexa 405 / Alexa 488 / Alexa 546 / Alexa 750

Goat anti rat: Alexa 405 / Alexa 488 / Alexa 546 / Alexa 750

3.1.8 Since P0 is present in myelin produced by all Schwann cells, can you propose a hypothesis to explain why there are MBP-positive but P0-negative myelin rings in the PNS roots of Dnm2 mutant animals? (Which cell type may be producing this myelin)? _____ _____. Discuss with your assistants.

3.1.9 Check the composition of the blocking buffer. Is it suitable for you to use a primary antibody produced in goat under these conditions? (Think of which species the secondary antibody had to target, discuss this issue with an assistant).

A:_____

3.1.10 Why do we use primary and secondary antibodies separately? Why not label the primary antibody instead?

A several secondary bodies bind to one primary antibody. therefore, we get a stronger signal and it is better visible in the analysis.

3.1.11 The three antibodies used for our staining were all produced in different animal species. Do you think this is a coincidence? If not, what could be the reason? A:_____

the animals do not have the protein normally and thus they produce antibodies against it. we use different species to obtain different antibodies, such that they can be labelled differently uniquely and we get different signals thus.

3.1.12 If you want to create a line of mice for conditional recombination exclusively in Schwann cells, which regulatory sequence would you place upstream of your Cre transgene?_____

the promoter of a myelinating protein exclusive to schwann cells (like P0). Cre will be after that promoter.

3.1.13 You suspect that your secondary antibody is unspecific. What would you do to exclude this possibility? A: add secondary antibody to other primary bodies. if it binds and has a signal, then it was unspecific

3.1.14 For most immunohistochemistry experiments, it is important to add a detergent, such as Triton X-100. Why? A: so that antibodies can cross the membranes

3.2 Slides with green dot – Sciatic nerves from control and mutant animals

3.2.1 Shoot the pictures of the slides you worked with (control and mutant sections) using 3 filter sets (when you look through the microscope oculars the fluorescence of each filter looks blue, green and red). – DO NOT MOVE THE SLIDE until all 3 colors are photographed in each section.

3.2.2 Remember, Glut 1 is labelled in blue, P0 in green, and CD45 in red. Check the schematic in page 7 to get a feeling for the location of these cells in a wildtype nerve.

3.2.3 Do you see any P0 (green) labelling in the mutant sections? Was it increased or reduced when compared to controls? A:_____. Can you propose a hypothesis to explain this observation? Hypothesis:_____ _____. To formulate a hypothesis, remember the semithin sections you looked at this morning, and remember the myelin stainings in the roots. Discuss with your assistants.

3.2.4 Does the localization of the cells marked in blue change in control and mutant samples? What is the change? A:_____.

3.2.5 Based on the observations, you can state that CD45 positive cells are **more** / **less** abundant in mutant nerves.

3.2.6 Immune cell presence is usually increased in peripheral nerves following injury, or in disease conditions. Do you think the PNS of Dnm2 mutant animals is healthy? **Yes**, **No**.

3.2.7 Can you conclude that Dnm2 expressed by Schwann cells **is important** / **is dispensable** for PNS myelination and **affects** / **does not affect** the behavior of other cell types resident in peripheral nerves.

For all fluorescence pictures acquired

3.3 Using Adobe Photoshop, **overlay the 3 images representing each antigen you are probing** in the "*Photoshop processing"* (starts on page 25). You can adjust each channel separately, and prepare an additional image with all 3 channels overlaid. If your immunostaining did not work, please use the default pictures located in the shared folder instead (see "Data Transfer" section on page 25 for instructions).

3.4 Using either Adobe Illustrator or Microsoft PowerPoint, **assemble the images you acquired into a data panel**. For the ground rules of panel assembly, and a step-by-step instructions of how to do this in Adobe Illustrator, please see the section "Assembling your pictures into a panel" (starts on page 30). You can also attend one the two tutorial sessions during the day. Each of you prepares a data panel with pictures of the sections you processed. One student per group prepares a panel with pictures from sections on the slide with the orange dot, and the colleague prepares a panel with pictures from sections on the slide with the green dot.

3.5 For the assembly of the sciatic nerve panel, you will also include electron micrographs present in the “Suter_GL_practicum” shared folder, inside another folder named “SN_EM” (see example panel on page 36).

3.6 You can find simple example panels derived from sections contained on both types of slides on pages 35 and 36 of this protocol. But they are not complete. Follow the instructions to assemble your complete panel.

3.7 Make sure to include a section with observations (results) and interpretation associated with your panel. The example panels also include examples for these sections, but you also need to complete them, or produce your own original ones.

When your panels are assembled, print them using the “p-hpl-f-20” printer (printer located on the F floor). Show your printed panels and your answers in the “**Results and Evaluation**” section of this protocol to the assistants to obtain your certification signature. Do not hesitate to ask questions, the assistants are here to help and teach you.

Microscopy and Image processing

Some of our microscopes are equipped with LED lamps, others with halogen lamps to provide illumination for fluorescence microscopy. **It is important that you do not turn off and on the halogen lamps on the microscopes located in D12**. The assistants will take care of this at the end of the practicum. Please listen carefully to the microscope introduction you will receive from the assistants, and do not hesitate to ask questions if you do not know what to do next in the microscope. Don’t press buttons you don’t understand, it may cause more harm than good.

Logging into the computers

All the computers should be logged in when you start working. But in case someone accidentally logs out, please use the credentials written in the laminated panel attached to each microscope:

Observing your samples with Fluorescence:

Make sure the fluorescence (also known as reflective) illumination is going through your samples. Also very important is to make sure that the transmission illumination is turned off.

1. Microscopes with halogen lamps: there is a “shutter” that you can move to block the light or allow it through to your sample. Open the shutter (see instructions from your assistant) and use the slide bar on the microscope to select the desired filter.

2. Microscopes with LED lamps: You need to **press inwards** a small black plastic wheel located on the right-side of the microscope. If you see a small blue LED light up right next to the wheel, then the power is on. Unlike the halogen lamps microscope, you have to press this wheel again to turn off the illumination. Use another large dented wheel at the front of the microscope to select the correct filter. Filter in position 1 is for transmission microscopy. Filter 2 corresponds to the blue channel, filter 3 to the green and filter 4 to the red channel.

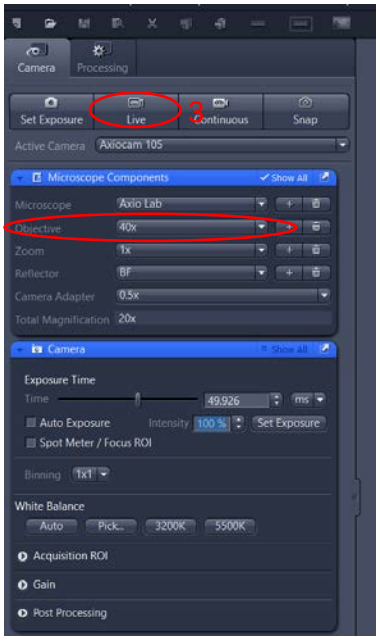
i. Focus your sample
ii. Browse through the different objectives and choose a good one to shoot your pictures (40x recommended).

Warning: The fluorophores will bleach if you leave them exposed to the fluorescent lamps for too long. This means you should adjust your settings, shoot you picture and then block the fluorescent light from reaching your sample. If you bleach the sample, you can no longer obtain a good signal for a picture.

Turning on the microscope/camera control software: Zeiss Zen

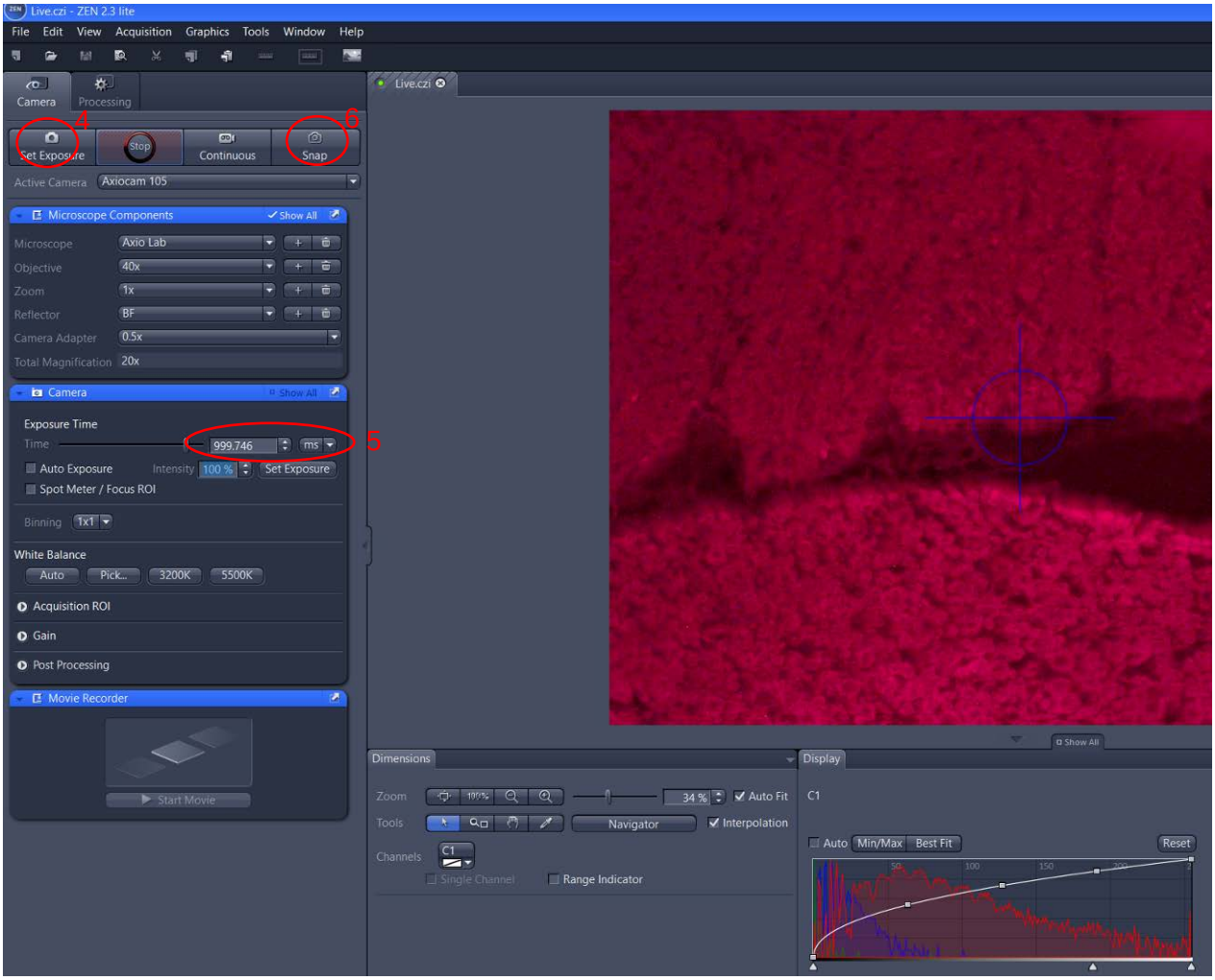
1. Click on the Zen Icon on your desktop.

2. When the software starts, **select the correct objective you are using from the dropdown menu** (1), otherwise you will get the wrong scale bar dimensions. The line that crosses the histogram should be on the default setting, **click "Reset"** (2) to make sure. Do not use the "Min/Max" or "Best Fit", they are not ideal to compare control to mutant samples. Please listen to the introduction by the assistant and don't hesitate to ask questions.



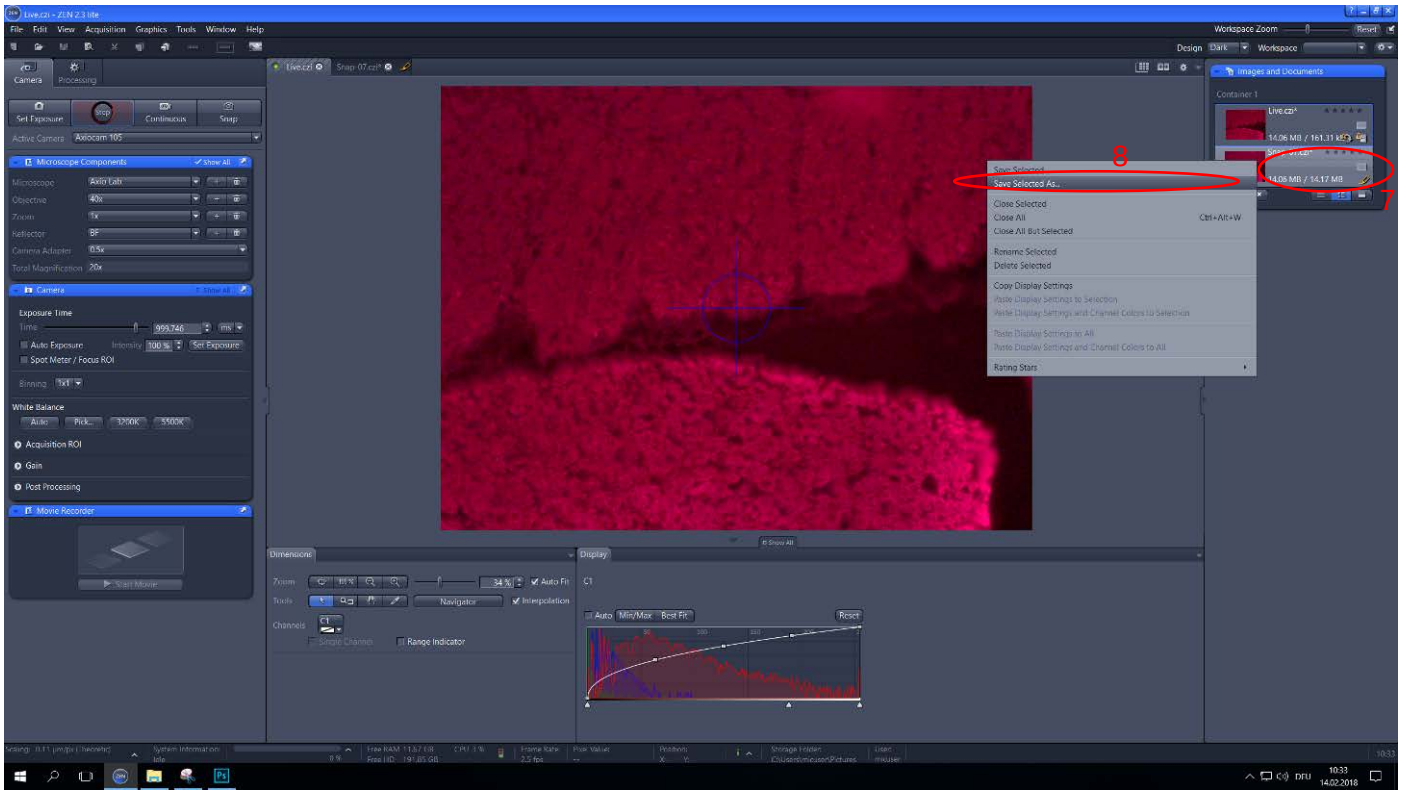
3. At this point you need to make sure that the illumination is going to the camera instead of the binoculars. There is a small switch in both microscopes you can use to redirect the light path. Listen to the instructions from the assistants for further details. Click on "Live" to see an image on the monitor (3).

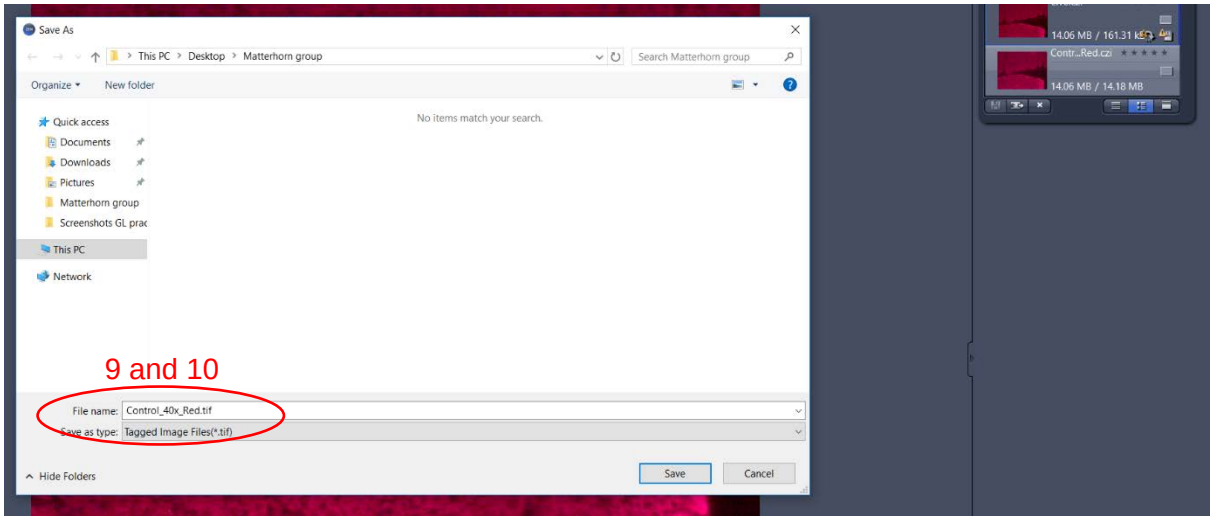
4. Press the “Set Exposure” button (4) to check the advice of the software for a good exposure time. In this case, the software advises approximately 1s (999.746 ms) (5). You may adjust this number, if you are unhappy with the advice of the software, by typing it in (5). Choose a good exposure for the channel and write down the value below. **You must use the same exposure value for the same channel (color) in control and mutant samples**!!! This is important to allow conclusions based on direct comparisons.



5. Set the reference exposure on the following samples, and then use the same value for the other genotype:
    a. Spinal cord roots: **exposure set for all 3 channels on control** sections (write the values below)
        i. Red Image: _____
        ii. Green Image: _____
        iii. Blue Image: _____
    b. Sciatic nerve slide: **P0 (green) – Set exposure on control** sections (exposure _____); **Glut1 (blue) – set exposure on mutant** sections (exposure _____) sections; **CD45 (red) – set exposure on mutant** sections (exposure _____).
6. When you are happy with your settings, hit “Snap” (6). This will shoot a digital picture for the channel you have selected. After you Snap a picture, it will show up on the Right-Side of your monitor.

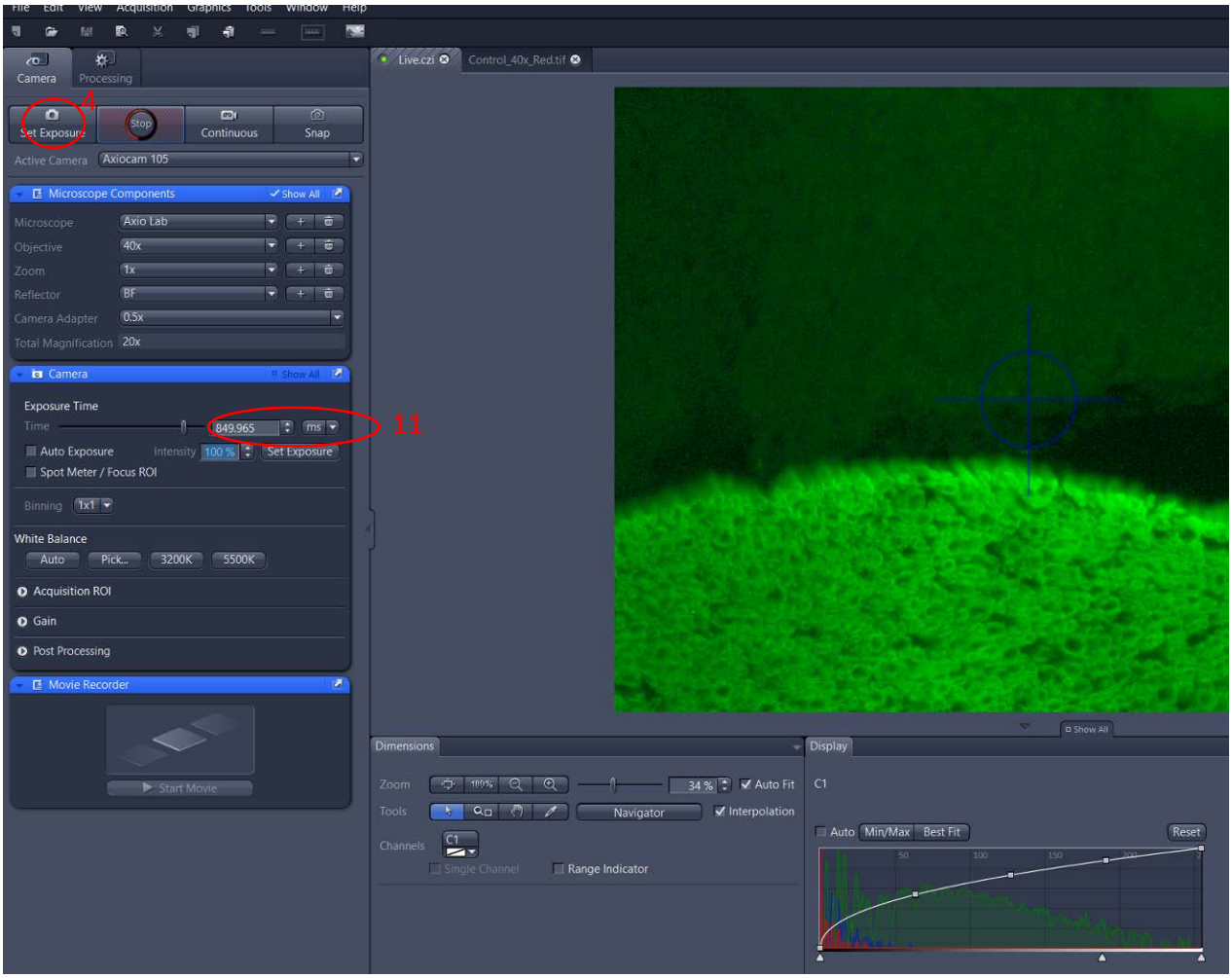
7. You can either save your image after snapping, or continue to acquire the other 2 channels and save everything when you are done. **But please do not move the sample until you have acquired all 3 channels.** To save the images, righ-click with your mouse button on top of the Snapped image (7), and left-click on “Save Selected As” (8), and create a folder in the desktop with your group name. Add relevant information to the image name, such as control or mutant, channel name or color, and objective used (9). Save the image in the Tagged Image File “.Tif” format (10).





8. When you change the filter to acquire the next channel, you have to click again on “Set Exposure” (4) and get a new optimal recommendation for your exposure (11). Most channels will have very different optimal exposure times. Note down which exposure you used for each individual channel on the first sample (see point 5 on previous page) and use the exact same values for the other sample. Depending on the intensity of your signal, fluorescence may be detected with an exposure between 25 ms

(milliseconds) and 5 s (seconds). Sometimes the software makes a wrong suggestion, but in most cases it is accurate.

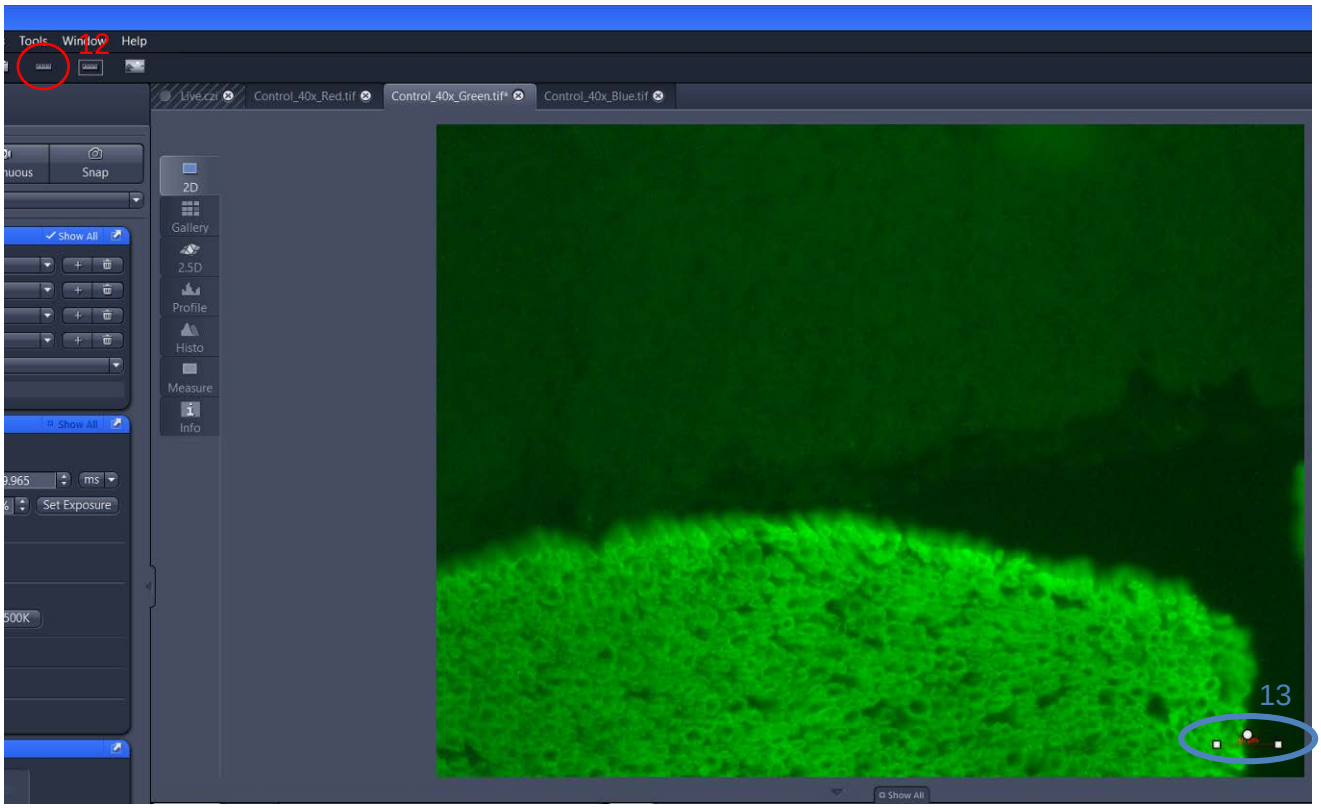


9. Snap and save your image, as explained on the previous page
10. Repeat the process to also acquire the blue channel, and save all the images. You can only move the stage after you acquired the images with the 3 different colors.

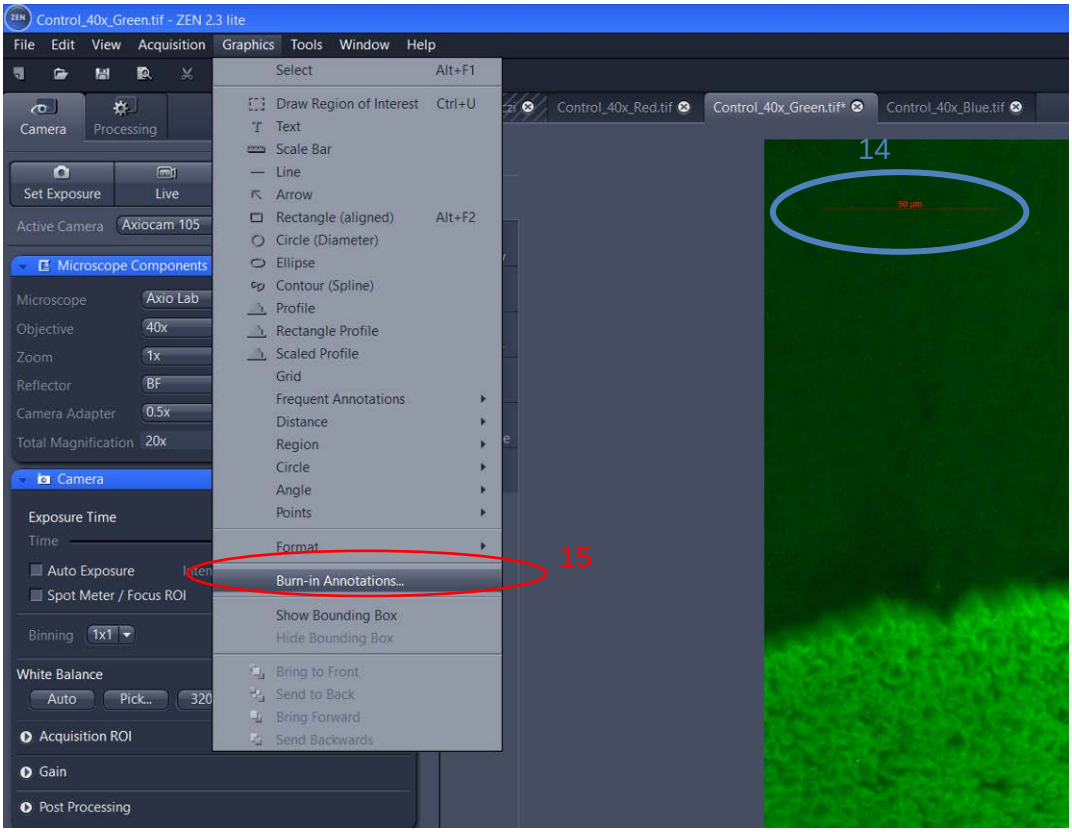
Placing and burning in the scale bar

You need to make sure that at least one of your images is saved with a scale bar. For this purpose you will follow the following steps and save a duplicated image (with scale bar).

11. With an image open, click on the scale bar icon (12). A scale bar will show up on the lower-right corner of the image (13). **Note:** Make sure you selected the correct objective on the software as shown on screenshot (1), Otherwise your scale bar will also be incorrect.



10. You can resize and drag the scale bar to any part of your image (14). Next you need to select the menu “Graphics” and click on “Burn-in Annotations…” (15). This will create a duplicated image with the scale bar imprinted on the image itself. You should save this duplicated image as a scale bar reference for the objective you used.



Acquiring transmission microscopy images:

11. To acquire transmission microscopy images, make sure the fluorescence (also known as reflective illumination) is blocked.
12. Turn on the transmission light, which should be a switch on the left-bottom side of the microscope
13. Adjust the light intensity with the regulation wheel located in the right-bottom side of the microscope
14. Direct the illumination to the camera and use “Set Exposure” to help you define a suitable exposure
15. Shoot your pictures and draw the scale bar as demonstrated above.

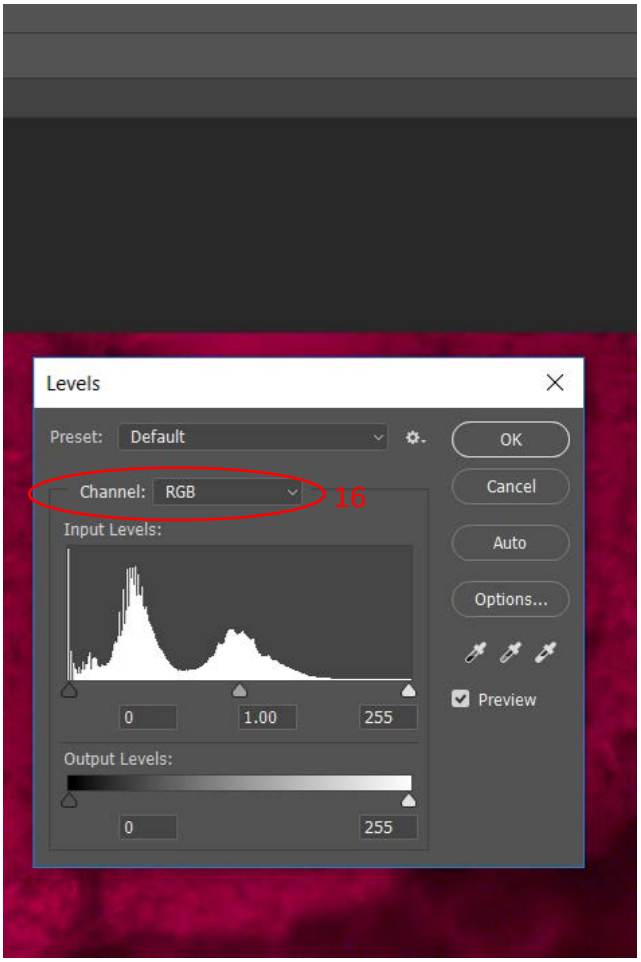
Data Transfer

Because 2 groups need to share 1 microscope (with 1 computer), the first group which obtains their pictures should transfer these pictures and continue working on another computer. There are several computers available in D12 and D23.1 for this purpose. To transfer your pictures, simply drag your folder into the shared folder named “Suter GL practicum”, which is already available in the desktop of each computer. If you have any questions, please seek help from an assistant. If your immunostaining did not work, use the pictures available in the shared folder called (default pictures). Start by **copying them into a folder with your name and then process them as instructed below**. Do not treat the original default pictures without copying them, as other people may need the originals too.

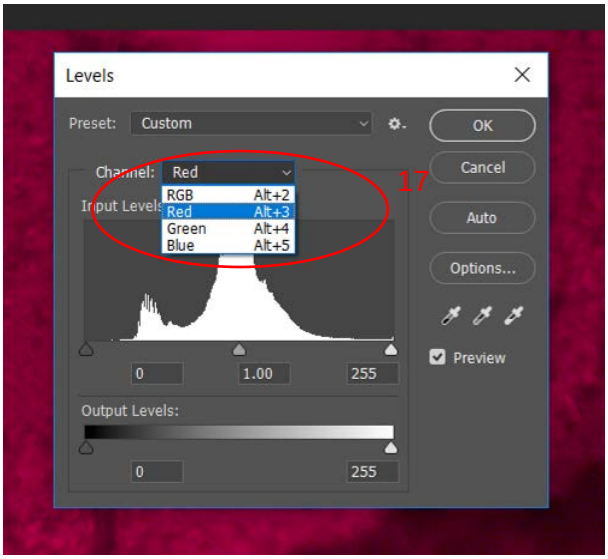
Photoshop processing

The next steps of image processing will be done using Adobe Photoshop.

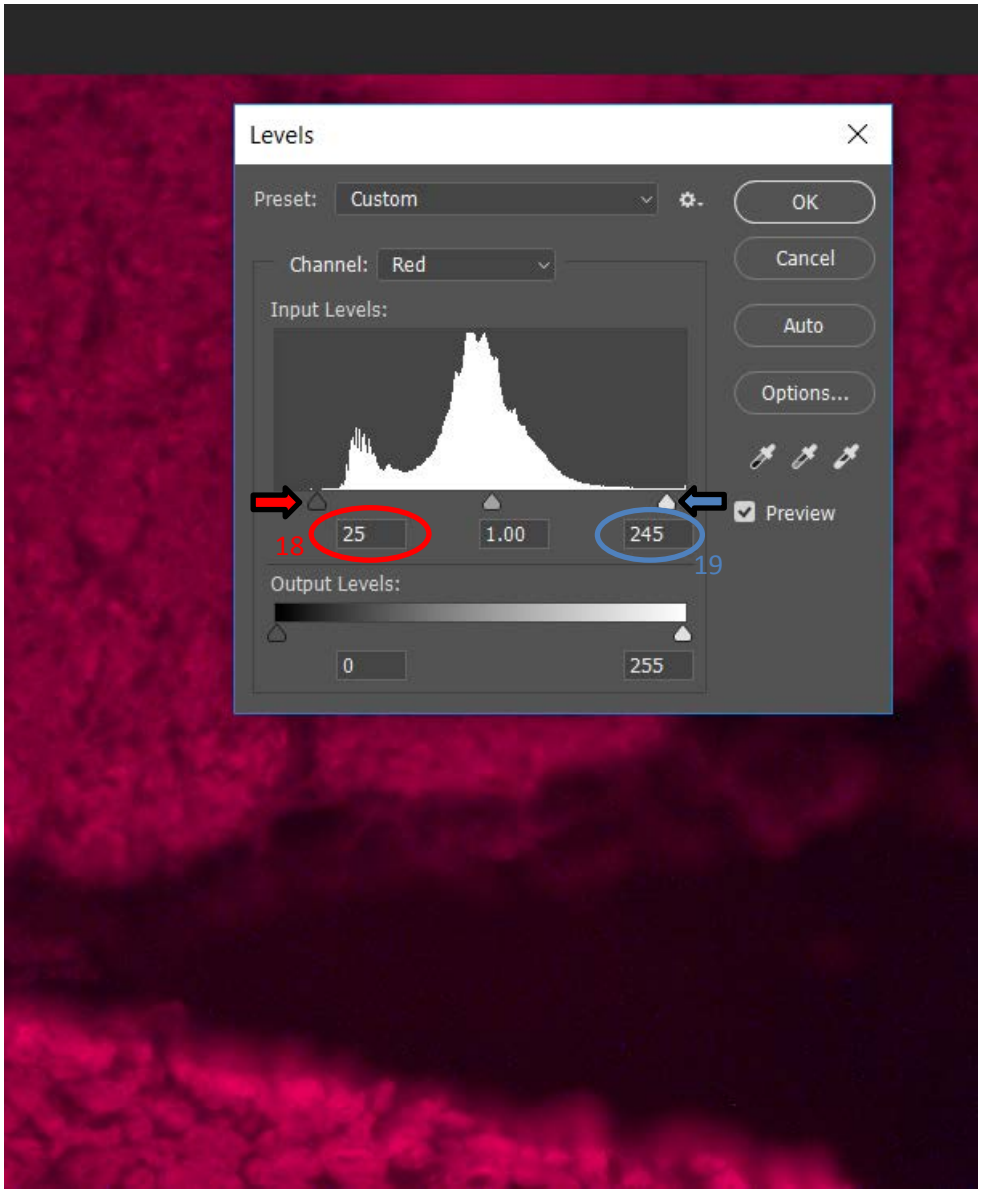
1. Start the Photoshop software
2. Open your images into Photoshop.
3. Consider as an example the Red Image. Select the red image on photoshop and click simultaneously on the keys **ctrl + L.** You can also use the menus, by selecting “Image”, “adjustments”, and “Levels”. You will get a level adjustment window as shown below. This window shows an histogram, and by default it will display the mixed RGB signal histogram (16).



4. You will now adjust levels of your images to discard “useless signals”. This will improve the quality of your pictures**, but make sure to process the control and mutant images of the same color in the same way, so you can compare them to each other**.
5. As you are processing the Red image, on the dropdown menu for “Channel”, select the Red Channel (17).



6. Drag the triangles near the edges of the histogram as shown (indicated by the red and the blue arrow) and remember the values displayed. The values go from 0 to 255, and in this example we set the background to “25” [encircled in red (18)] and we increased the signal to “245” [encircled in blue (19)]. You can also write down these values directly instead of dragging the triangles. Because you want to compare 2 samples (a control and a mutant) to each other, you should find a good balance for your signal on one of the samples and **simply write the exact same values on the pictures for the other sample**. See point 7 below for which sample to use as primary reference.



7. On which sample should you set the values? Use the following:

    a. Spinal cord roots: **Set the values for the Red, for the Green, and for the Blue** images on **Control samples** (write the values below). In the example above, the *Red control image would be noted as (25-245)*. The same values would be used for the mutant Red image.

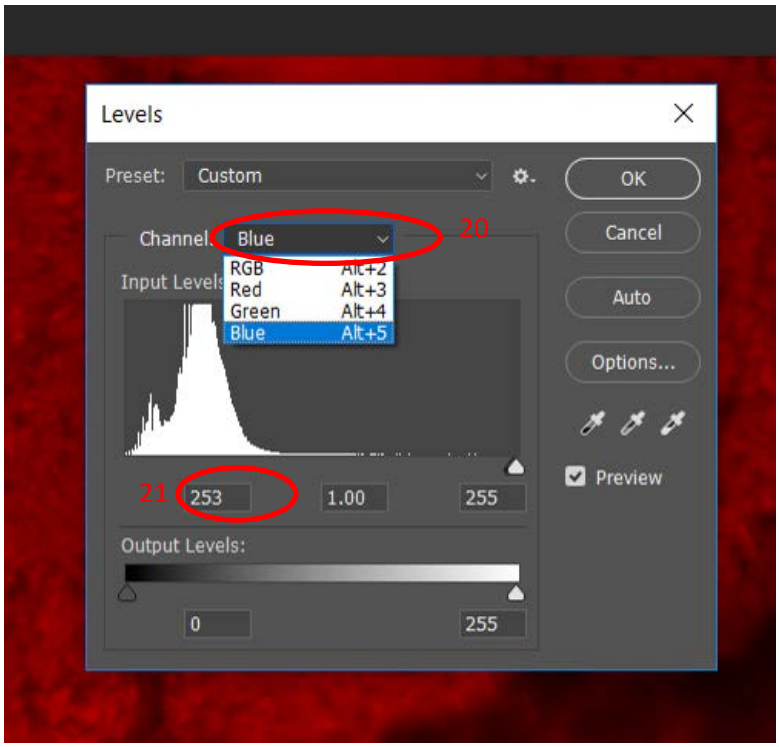
        i. Red Image: _____

        ii. Green Image: _____

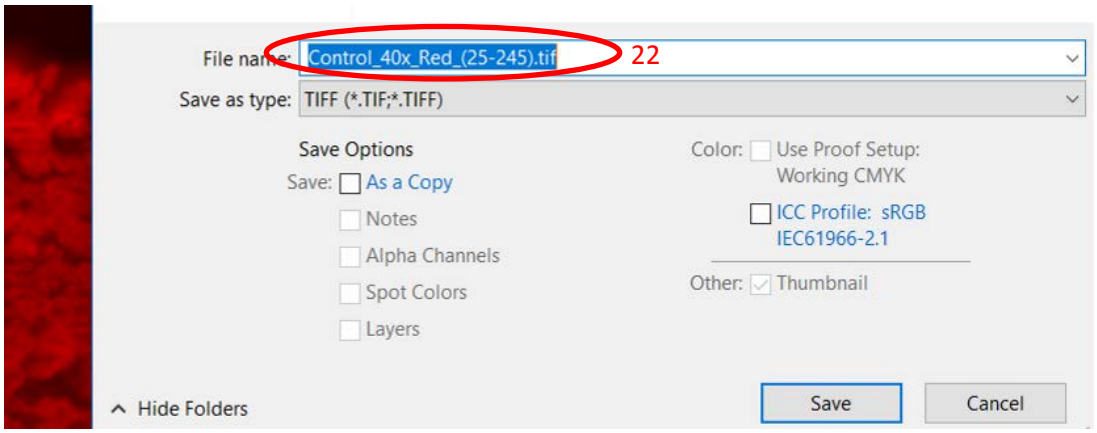
        iii. Blue Image: _____

b. Sciatic nerve slide: **P0 – Set levels on control** sections (Green _____);
Glut1 – set exposure on mutant sections (Blue _____);
CD45 – set exposure on mutant sections (Red _____).

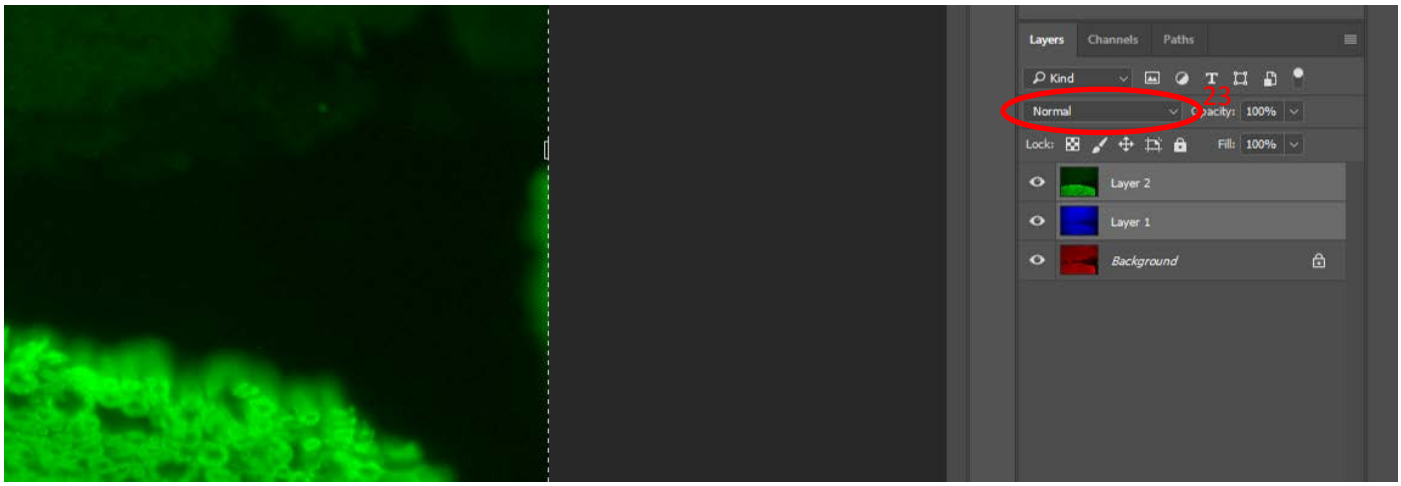
8. Before you are done with the Red image, you need to eliminate the green channel and the Blue channel from the Red image. To do that, select the Blue channel (20) and write “253” on the background subtraction field (21).



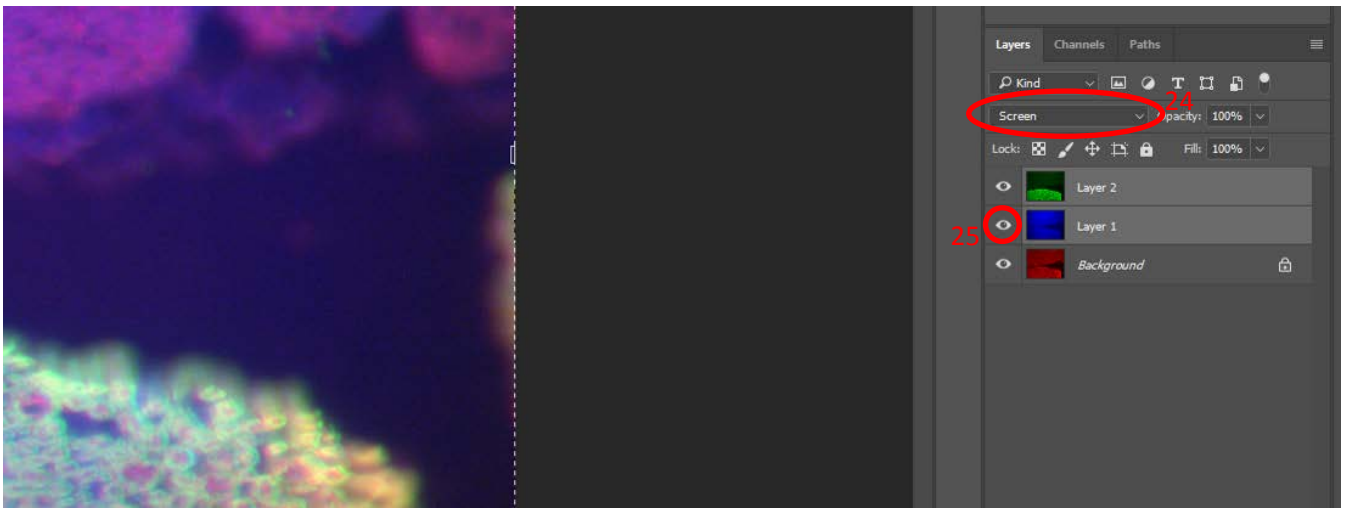
9. Finally, select the green channel and eliminate the signal in it as well, using the procedure described on the previous step. You should now have a purely Red image, without junk signals on the green and the blue channels.
10. You can now save this image with a new name. A good strategy is to write the adjustment you did on the name of the image (22). However, do not replace your original one, it is always a good idea to preserve your original data. Sometimes your peers or scientific journals really want to see the original unprocessed pictures.



11. You should now go back to step 3 and do the same procedure for the Green and the Blue images you photographed at the microscope.
12. Remember:
    a. On the Blue image, you will adjust the optimal Blue signal as demonstrated on step 6, and you will discard the Red and Green signals.
    b. On the Green image, you will adjust the optimal Green signal as demonstrated on step 6, and you will discard the Red and Blue signals.
13. Once you have your 3 images ready and saved, it is time to overlay them. To do this, select an image (for example the blue) and hit simultaneously “Ctrl + A”. This will select the whole picture.
14. Then select “Ctrl + C” to copy it.
15. Paste the image on top of another channel (for example the red). This will create a new layer with the freshly pasted picture.
16. Do this also for the green image.
17. You now have 2 layers on top of the background layer, but you only see the latest image you pasted (in this case, the green).



18. To see all the layers overlaid on each other, you must select the 2 layers on top of the background, and then click on the dropdown menu (23) on the “Layers” window and click on the option “Screen” (24).You can also set this option layer by layer. Congratulations, you can now see all your signals detected by the various antibodies overlaid on each other.



19. Save your image in “tif” format and preserve the layers (do not flatten the image). To save images with only 2 channels visible at a time, just click on the eye icon next to each layer/color encircled in red (25) to show or hide them from view. If you save images while viewing only some of the layers they will be imported that way to various programs.

Assembling your pictures into a panel

1. Show your image with an overlay of all channels (as created above) AND show also the images of every single channel individually (see panel example on pages 35 and 36)
2. Don’t forget to include the scale bar on at least one image for each different magnification in the same panel.
3. Label every image with the name of the detected protein, and label which pictures were taken from control and mutant sections.
4. When you place markings (arrowheads, arrows, asterisks, etc.) to highlight structures on your images, make sure to place the marking on the images for the individual channels plus on the overlay image, and not just on one of them (see example panels).
5. Align your images in a consistent manner to each other. Use the alignment tools of the panel assembly software (available in both PowerPoint and illustrator).
6. Every panel/figure is coupled to a figure legend which states the plain observations of the figure, which are the **results**, without too much interpretation or discussion. Look at the example panels in page 35 and 36 and complete the results section with the keywords available (or produce your own statement of the results).
7. The **interpretation** of the results is also important. But the interpretation goes beyond the plain observation and you should be able to distinguish these 2 elements. In the example panels (pages 35 and 36) you can also find an interpretation section with incomplete sentences which you can either fill in the gaps, or you can write the interpretation in your own words.

When your panels are assembled, print them using the “p-hpl-f-20” printer. Show your printed panels and your answers in the “**Results and Evaluation**” section of this protocol to the assistants to obtain your certification signature. Do not hesitate to ask questions, the assistants are here to help and teach you

Adobe Illustrator is an excellent program to assemble your images into a figure panel. You may use it if you are familiar with it already and you can follow some guiding steps in the next pages, but due to time limitations and the complexity of illustrator, you can also assemble your panels using Microsoft PowerPoint.

General instructions for Microsoft PowerPoint:

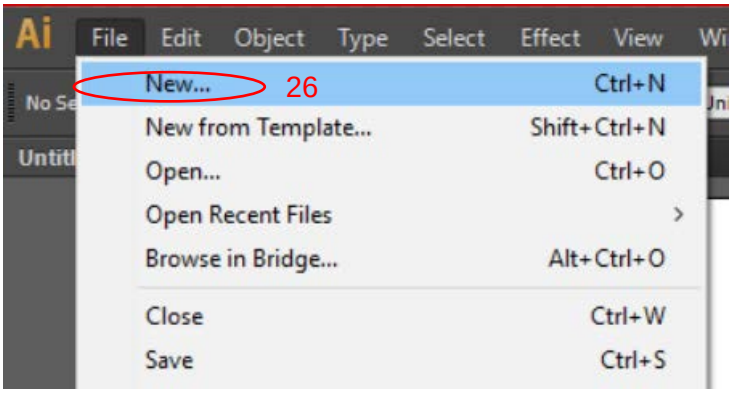
1. Set the slide size in PowerPoint to A4 (“Design” tab – “Page Setup” button – Set size to A4). You can use portrait or landscape, but landscape may be more convenient for your panel.

2. You are probably already familiar with Microsoft PowerPoint and its various tools, which you can use to assemble your panel. If you have questions please ask your nearest assistant.

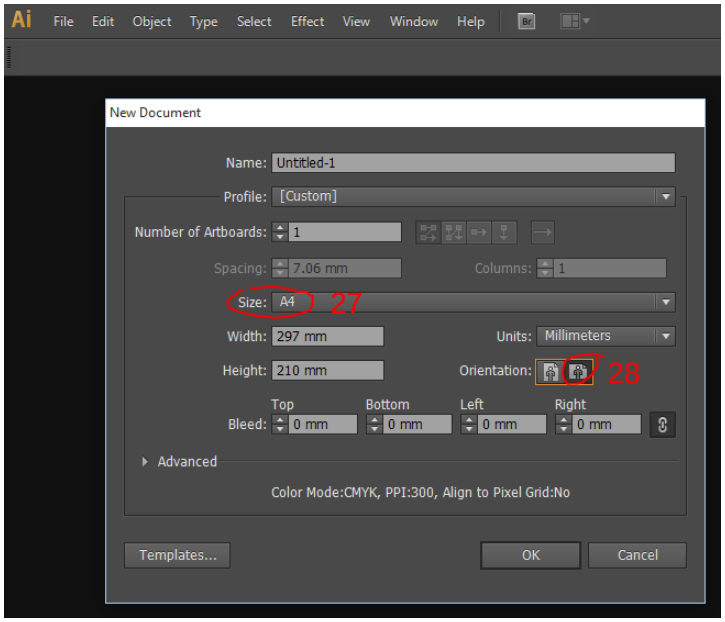
Step by step instructions for basic tools to assemble your panels in Adobe Illustrator:

You can attend the Tutorial lecture provided in 2 sessions during the day, in addition to this guide

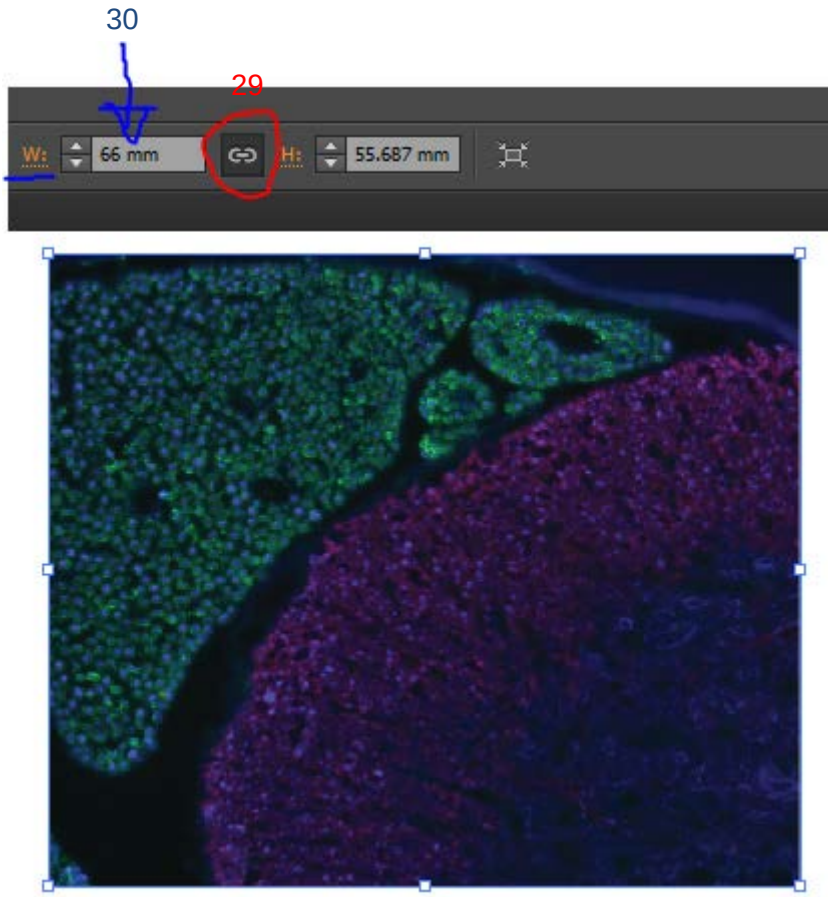
1. Open Adobe Illustrator (available on every computer of your practical course). Click on “File” and select “New” (26)



2. On the size select A4 (27). On the orientation, select landscape (28).

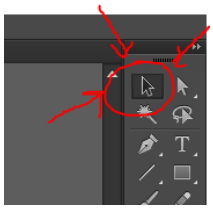


3. You can easily zoom in and out of your artboard by holding down “alt” while scrolling with your mouse weal or touchpad scroll. It is also easy to move side to side, by holding down “ctrl” while scrolling (with the mouse weal or touchpad scroll).
4. You can copy-paste your images directly from Photoshop. This is the fastest option. To do so, select your image on Photoshop, press the keys “ctrl+A” followed by “ctrl+C”. This selects your entire image and copies it.
5. Go back to illustrator and press the keys “ctrl+V”. Your image is now pasted in illustrator, but it is probably too big. Click on the image and look at the dimensions on the top bar. Link the width (W) and height (H) by pressing the chain icon (29). For the SC/roots panel, set the maximum width to 66mm (30). For the SN panel, set it to 52 mm. The height will be automatically scaled proportionally.

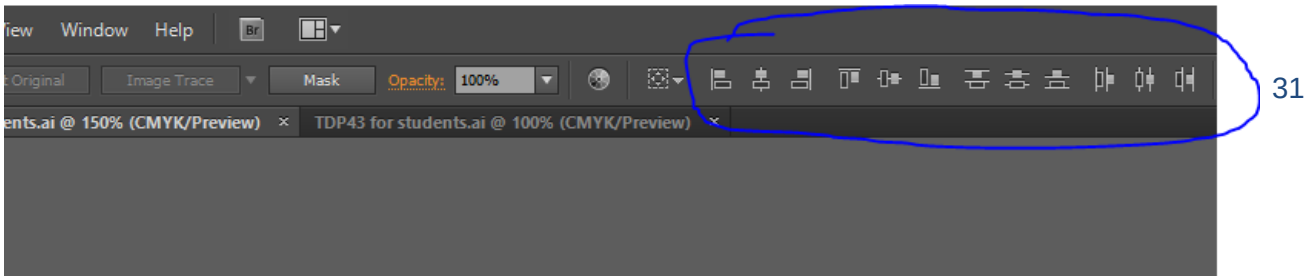


6. Repeat this procedure to import and scale all your images to illustrator.

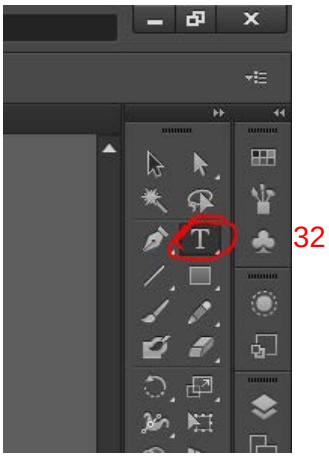
7. Drag the images inside the artboard (the white area) and align them with the alignment tool. To do so, hold down “shift” plus clicking, or by drawing a square, and select all the images you want to align to each other (while having the **selection tool activated, see screenshot below**).



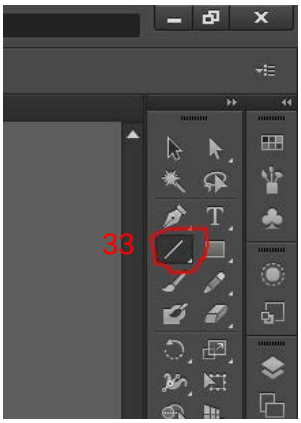
8. When you select various items, the align options should automatically become visible (31) at the top of the monitor. If they don’t, make them visible by selecting “window” and clicking on “Align”.



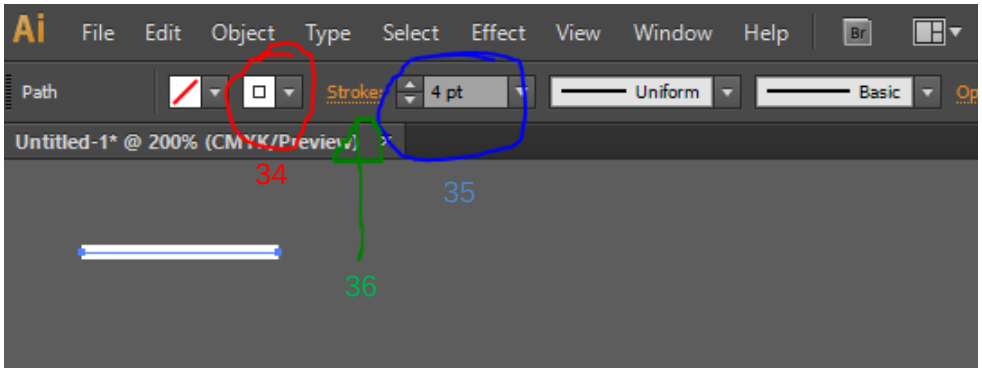
9. To place text labels, use the text tool (32). If you can't see the tools on your monitor, click on "window" and select "tools".



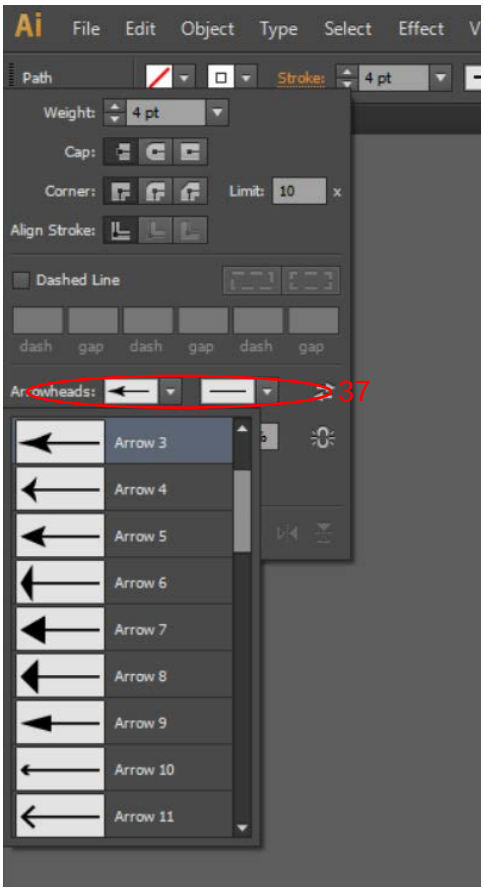
10. Don't forget to also import your image with the scale bar and scaling it to the same level as the other images. To draw a line of the same size as your scale, use the path tool (33).



11. Hold down shift while you draw the path to make it perfectly straight. You can set the color of the path (34) (in this case, white) and the thickness (35) (in this case, 4pt). If you click Stroke (36, see green arrow), you get a lot more options to create dashed lines, arrows, etc.



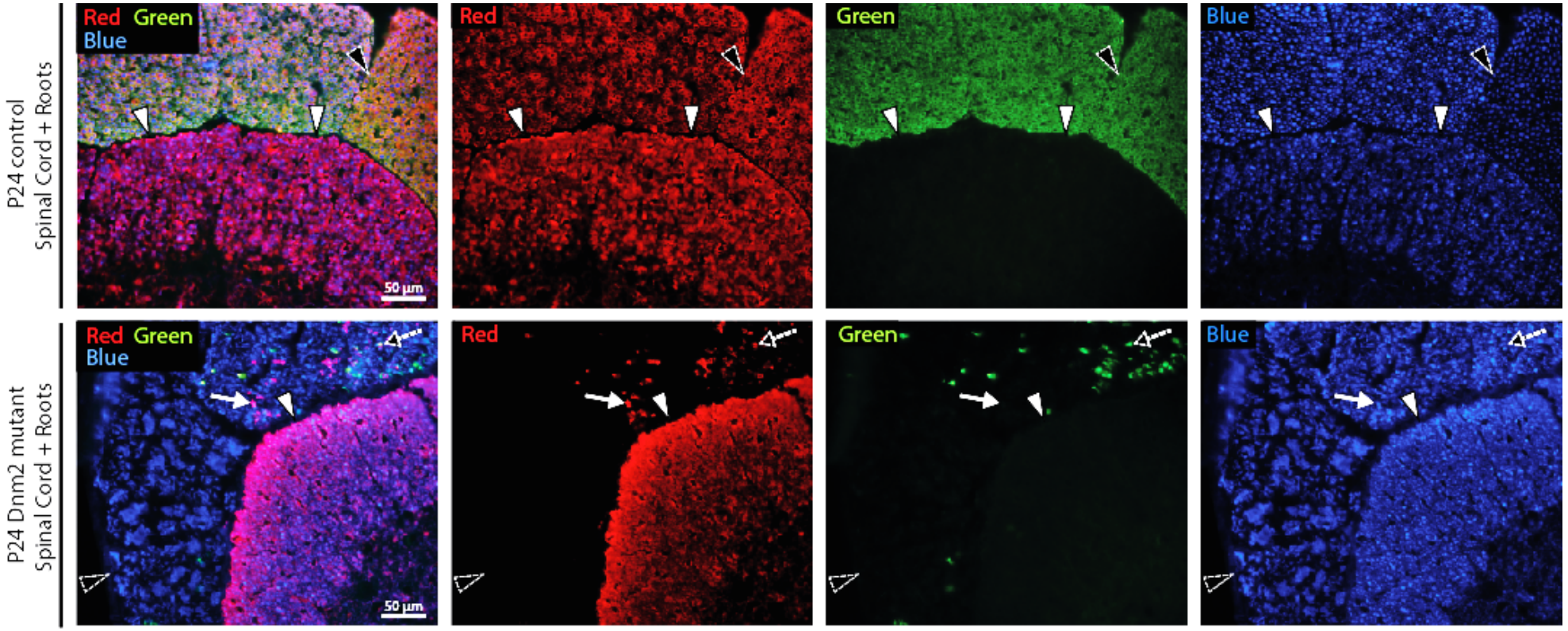
12. Arrows are also created with the path tool. First draw a path as before, then select "Stroke" and click the dropdown menu "arrowheads" (37).



13. You can use arrows or arrowheads to mark relevant structures on your pictures. Just remember to add them at the same position in every channel of your image.

14. Don’t forget to save your file regularly, so you don’t lose your work if the program crashes. If you have any questions, please ask your nearest assistant.

If you want, look at some example panels on pages 35 and 36 of this protocol. Do not forget to create a legend with “Results” and “Interpretation”, or to fill the text gaps in pages 35 and 36, and to print your panel at the end. You can use the “p-hpl-f-20” printer. Please show your printed panel and the answers to the questions in the **“Results and Evaluation”** section of this protocol to get your signature from an assistant.



Note: replace the "red", "green", and "blue" in the pictures above by the name of the antigen detected with each fluorescense channel.

Results:

Figure 1 - Deletion of Dnm2 in Schwann cells leads to a reduced myelination in peripheral nerve roots. Cryosections (10 µm) derived from control and Dnm2 mutant samples at P24 were immunolabelled with antibodies specifically recognizing myelin basic protein (MBP), myelin protein zero (P0) and neurofilament (NF). White arrowheads indicate_____, and black arrowhead indicates_____. _____ and _____ can both be detected in the PNS, whereas _____ is not detected in the CNS. The number of myelin rings in the PNS roots marked by MBP and P0 (black arrow with white dashed contour) is reduced in mutant sections when compared to controls. Furthermore, mutant PNS roots display myelin rings that are _____ for MBP but negative for _____ (white arrow).

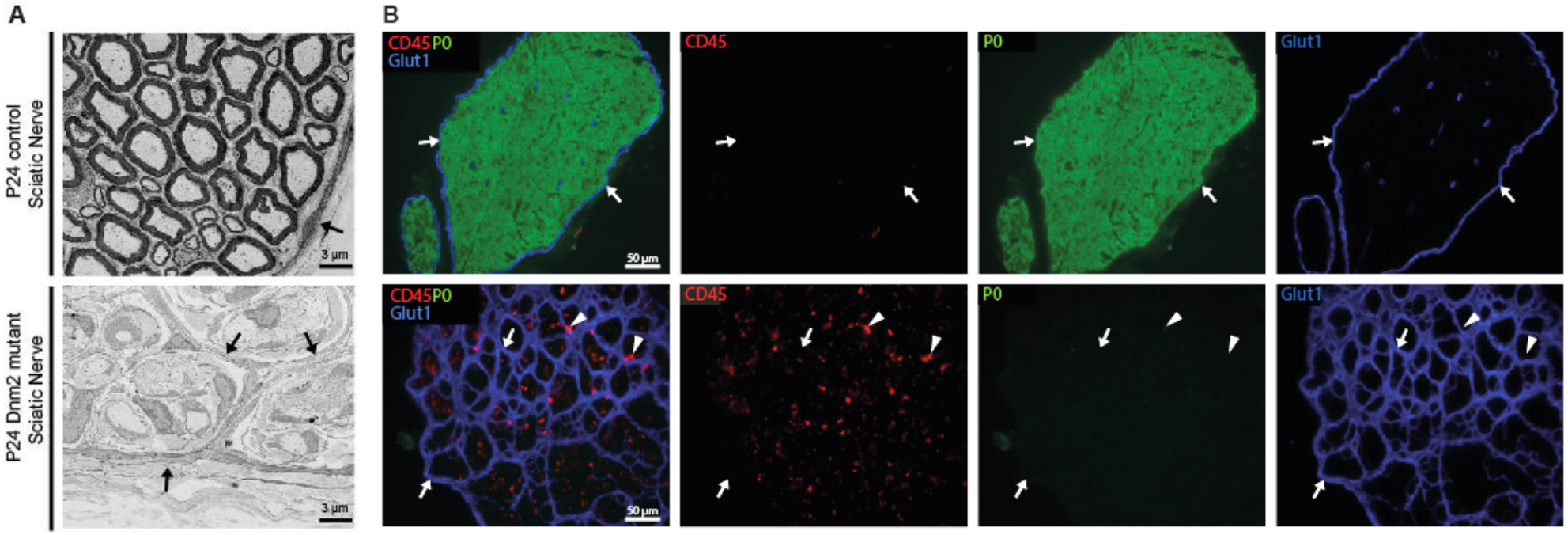
keywords: PNS roots, spinal cord, P0, MBP, positive.

Interpretation:

These observations suggest that Dnm2 acts to _____ myelination in _____.

The presence of _____ positive and _____ negative myelin rings in the PNS roots of Dnm2 mutant samples suggests that _____ might have migrated out of the CNS and invaded the PNS roots.

keywords: PNS roots, oligodendrocytes, promote, P0, MBP.



Results:

Figure 2 - Deletion of Dnm2 in Schwann cells leads to a reduced myelination and altered cellularity of sciatic nerves. **A)** Electron micrographs (EMs) of ultrathin sections derived from sciatic nerve of control and Dnm2 mutant samples at P24. The images represent the same structures observed in semithin sections during the practical course, but with greater resolution. Note the elongated perineurial cells which surround the nerve in _____ samples (black arrow, top panel); which are less compacted and penetrate inside the peripheral nerve in the _____ samples (black arrows, bottom). Note the severe _____ of dark-colored myelin rings surrounding the axons in the mutant samples. **B)** Cryosections (10 µm) derived from control and Dnm2 mutant samples at P24 were immunolabelled with antibodies specifically recognizing the immune cell marker CD45, myelin protein zero (P0) and the perineurial and endothelial cell marker Glut1. White arrowheads indicate_____, and white arrows point to_____. The number of P0 positive structures are strongly _____ in _____ samples, which is coherent with the observations in electron micrographs. The Glut1 positive elongated structures can be detected around and _____ the sciatic nerve in the mutant samples, whereas these elongated cells are restricted to the perineurial surrounding layer in control samples. The few Glut 1-positive small structures visible in controls are likely to be _____ cells from endoneurial blood vessels. Cells expressing the CD45 immune lineage marker were present in _____ but not in _____ samples.

Interpretation:

These observations suggest that Dnm2 acts to _____ myelination in sciatic nerves. This role is _____ to what was observed in peripheral nerve roots. The elongated cells observed in the endoneurium of Dnm2 mutants on semithin and EMs are correlated with increased Glut1 immunostaining, and are therefore most likely _____ cells. It is known that perineurial cells can occupy the endoneurium in the event of Schwann cell apoptosis. Apoptosis within peripheral nerves is also likely to promote expansion of the immune cell population in the nerve to clean up cell debris. Consistently, there is a marked _____ of immune cell presence specifically in the _____nerves. It is therefore possible that Dnm2 protects Schwann cell survival, but this needs to be tested with additional experiments to detect apoptosis specifically in Schwann cells.

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Myelin Protein 0 (P0) antibody (AB9352)

Glut1 antibody (07-1401)

CD45 antibody (550539)

Myelin Basic Protein (MBP) antibody (MCA409S)

Goat anti mouse Alexa 405-conjugated antibody (A31553)

Goat anti rabbit Alexa 405-conjugated antibody (A31556)

Goat anti rat Alexa 546-conjugated antibody (A11081)

Donkey anti chicken Alexa 488-conjugated (703-545-155)