IMMUNOFLUORESCEENCE STAINING OF SUBCELLULAR STRUCTURES IN PORCINE TRIGEMINAL GANGLIA CELLS

by

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**Summary**

Alphaherpesviruses such as pseudorabies virus (PRV) are known for their ability to use the microtubule transport system of neurons to migrate within nerve fibers. PRV gains access to the nervous system of its host by entering sensory nerve endings originating from pseudounipolar neurons. Little is known about the organization of the microtubule transport system of pseudounipolar neurons. To gain further insight into this specific pathomechanism of PRV we optimized immunofluorescence (IF) stainings of thin sections of porcine trigeminal ganglia (TGs) to detect different intracellular structures. Several subsequent optimalization steps resulted in creating a fixation and staining method in which the morphology of the cells as well as subcellular structures are preserved. To identify whether cell cultures are comparable to the *in vivo* situation we also performed IF staining on porcine TG cultures. Based on these stainings, different molecules, such as neurofilament and alpha- or beta-III-tubulin, appear to be present in the same conformation in cell cultures as in thin sections. Other neuronal characteristics, such as nerve branching, differed *in vitro* cultured TG neurons versus thin sections. In conclusion, we have optimized a fixation and staining protocol that may aid in determining the microtubule transport system of pseudounipolar neurons, and its use by alphaherpesviruses.

**Key Words:** Pseudorabies - Microtubules - Pseudounipolar neuron - Trigeminal ganglion
Abbreviations
Ab, Antibody
CNS, Central Nervous System
FSG, Fishskin Gelatine
IF, Immunofluorescence
KRP, Kinesin-Related Protein
MAP, Microtubule-Associated Protein
MT, Microtubule
MTOC, Microtubule-Organizing Center
MTS, Microtubule Transport System
NF200, Neurofilament 200
NF68, Neurofilament 68
NFM, Neurofilament M
NGS, Negative Goat Serum
PNS, Peripheral Nervous System
PUN, Pseudounipolar Neuron
T20, Tween 20
TR, Texas Red
TX100, Triton-X-100
γ-TuRC, gamma-Tubulin Ring Complex
1. Introduction

One of the systems that enable a cell to move organelles and thereby sustain its vital functions is the microtubules transport system. But not only the cell itself, but also a variety of pathogens have succeeded to use this transport system. As described in an earlier manuscript, herpesviruses are strongly suspected to use the MT transport system to move along axons and dendrites, thereby providing a mean to the virus to efficiently engage in long distance transport [37]. Pseudorabies virus or suid herpes virus 1 (SHV-1) is an alphaherpesvirus which has been present in the European pig population for a long time and is still widely used in laboratory research, to study the infection and latency characteristics of herpesviruses. Herpesviruses gain access to the nervous system of an animal by sensory nerve endings in the skin. These nerve endings originate from pseudounipolar neurons (PUNs). PUNs are found in different ganglia. For several alphaherpesviruses including PRV but also herpes simplex virus I and bovine herpesvirus I, neurons of the trigeminal ganglion (TG) are a major target cell type. To be able to further investigate the transport of these viruses within axons, we wanted to refine immunofluorescence (IF) staining on porcine trigeminal ganglia (TG). Not only to get a first impression of the MTs in these cells, but also to have a reliable protocol for future investigations. Further, we wish to compare the IF staining of MTs on tissue sections with the one of in vitro cultured TG neurons, to identify whether significant differences would be observed.

1.1 The pseudounipolar neuron

The nervous system is a complex construction of different neuronal and non-neuronal cells, each highly differentiated to fulfill certain tasks. The basic structure of a neuron consists of a cell body with dendrites and an axon. The dendrites are responsible for the “signal input” whereas at the axon hillock a signal gets generated and conducted, for example, a synapse or an endplate. Depending on location and function neurons differ in morphology and molecular structure (Fig.2). In the eye for example, unipolar, bipolar, horizontal, amacrine and ganglion cells are found. Each of these highly differentiated neuronal cells is working together to make sight possible. In the cortex of the brain, special pyramid cells, displaying multiple dendrites, connect the different layers of the cortex [26]. PUNs are found in sensory ganglia of certain cranial nerves (nervus trigeminus, n.fascialis, n.glossopharyngeus, n.vagus) and the sensory spinal ganglia: the dorsal root ganglia (DRGs) [3, 25-26]. PUNs are responsible for the sensible innervations of the skin, mucosae, hair follicles and muscles [30]. PUNs originate from bipolar nerve cells in ganglia. One prolongation grows into the spinal cord, which will develop to be an axon. The other prolongation will migrate with muscle and skin assets to the periphery to become a dendrite or sensible nerve ending [26]. During the development of the PUN the appendices of the axon and the dendrite fuse at their origin, creating only one
extension at the cell body [26]. The dendrite changes its morphological and functional features to those of an axon, and turns into a so called “dendritic axon” [26]. This is illustrated in figure 1. The PUN is the only known nerve cell which possesses a straight connection from the innervation site to the synapse with the following neuron [26]. Because of this metamorphosis the PUNs hold the unique capacity not to regenerate the electric signal at their axon hillock but allowing it to pass straight through to the synapse, circumventing the cell body [26]. Like other neuronal cells, only little is known about the special features of the PUNs. There has been little research concerning the characteristics of the dendritic axon of PUNs as well as the effect of the “by-passing” of the cell body onto the morphological and molecular characteristics of these neurons.

1.2 The microtubule transport system in neurons

Neuronal cells have a well differentiated microtubule transport system (MTS), allowing for example the distribution of mitochondria along the axon as well as the delivery of neurotransmitters to synapses [1].

1.2.1 Microtubules

Microtubules form the “transport highway” within an axon or a dendrite. A MT is formed by 13 protofilaments, which are arranged in a parallel fashion, forming the wall of a hollow cylinder. The protofilaments consist of alpha- and beta-tubulin dimers, which are connected through GTP molecules. The diameter of a MT is 25nm [1, 19, 26]. Due to the orientation of the tubulin dimers one end of the MT only consists of alpha-tubulin, called the slow growing “minus end”. The end presenting beta-tubulin is named the “plus end”, because of its ability to align new tubulin dimers and thereby contributing to the fast growth of the MT. At the “plus end” of the MT there is a constant struggle between destruction and polymerization. This is due to GTP molecules, which stabilize the plus end only temporarily. If there are no new tubulin dimers attached (rescue), the hydrolysis of GTP to GDP in the tubulin monomers results in destruction (catastrophe) of the MT, beginning at the plus end [11, 22]. All the MTs emerge from one location within the cell: the microtubule-organizing center (MTOC) or centrosome. The MTOC consists of two centrioles, which are aligned in a 90° angle to each other, gamma-tubulin ring complexes (γ-TuRCs) and other pericentriolar material. The “minus ends” of the MTs are centered within the MTOC, where they are bound to the γ-

![Fig. 3: Schematic drawing according to [1, 26]. The MTOC is the point of origin of all MTs within a cell. The MTs are fixed with their “minus end” onto γ-TuRCs, which probably serve as templates. The center of the MTOC is formed by two centrioles, which are surrounded by pericentriolar material.](image-url)
TuRCs. It is thought that the γ-TuRC serves as a template for the MT with its 13 protofilaments [1, 40-41]. The MTOC and its features are illustrated in Fig. 3. The organization of MTs in neurons differs in many ways from other cell types. The “plus end” is only pointing away from the cell body in axons and distal dendrites, whereas in proximal dendrites they are of mixed polarity [19]. The MTs are also differently organized in axons versus dendrites. This is mainly due to microtubule-associated proteins (MAPs). MAPs determine the stability and dynamics of MTs. The MAP1 family for example, is known for its role in stabilization of MTs and the development of the brain [15]. MAPs also determine the spacing between different MTs. The spacing in axons is typically 20nm, where mainly tau (an axonal MAP) is expressed. In dendrites the space between two MTs is 65nm, due to the overexpression of MAP2 [10, 19, 26].

1.2.2 Molecular motors
In neuronal cells, like in most eukaryotic cells, there are primarily two different molecular motors: kinesin and dynein. They are called molecular motors, due to their ability to bind transport cargo and move along MTs. Dynein is considered to be mainly responsible for the retrograde or “minus end” transport, whereas kinesin is primarily found moving anterogradely towards the “plus end” of the MT. Nevertheless, dynein as well as kinesin can be found moving anterogradely respectively retrogradely [16]. Dynein possesses a “dynactin complex” which can bind a variety of cargoes, whereas kinesin has a number of different family members, each varying in structure [17]. The kinesin superfamily is divided into different families of kinesin-related proteins (KRP) [16-18]. The basic structure of kinesin consists of two heavy chains and two light chains per active motor, two globular head motor domains and an elongated coiled-coil responsible for heavy chain dimerization. This structure can vary within the KRP [16]. Cytoplasmatic dyneins are composed of two heavy chains, including two large motor domains and a large and variable number of associated light chains [17, 39]. The structure of kinesin and dynein is illustrated in Fig. 4 and Fig. 5. The movement of these molecular motors is based on the hydrolysis of ATP. The hydrolysis induces a conformational change, which carries one of the heavy chain heads forward to the next tubulin dimer on the MT [14, 38]. The organelles transported are membrane-enclosed and kinesin is able to bind them through membrane-associated motor receptors. Dynein binds to these cargo vesicles, as noted earlier, by the use of a “dynactin complex” (Fig. 5) [1, 17, 39].

1.2.3 The microtubule transport system in pseudounipolar neurons
As pointed out in the first section of the introduction, only little is known about PUNs. The MTS has been studied in non-neuronal cells and in in vitro cultures of neurons. But none of these neurons exhibited the special features of PUNs. Since the cell body in PUNs is bypassed by the direct connection of the axon and the dendritic axon, one could speculate whether the MTs also form a direct connection between the innervations site and the synapse, or originate like in other cells close to the nucleus. A first step to analyze the organization of MTs in PUNs would be, for example, IF imaging of the MTs in a TG. The detection of the location of the MTOC within PUNs could also give further information on this subject.
2. Material and Methods

2.1. Immunofluorescence (IF) staining of porcine trigeminal ganglia

Excision and Freezing

Euthanisation of 3 months (Exp. I, II), 4 months (Exp. III, IV, VI), 7 weeks (Exp. IV, V, VI), 3 weeks (Exp. VII, VIII) old pigs with sodiumpentobarbital 20% (Kela) and excision of trigeminal ganglia (TGs).

Two techniques were used to prepare them for sectioning:

A. Fresh frozen: TGs were put into small tubes and submerged in -40°C dry-ice cooled 70% ethanol (Exp. I, II) or isopropanol (VWR) (Exp. III, IV, VI).

B. Pre-fixation: TGs were first submerged in 4% (Exp. IV, V, VI, VIII) or 3% (Exp. VIII) paraformaldehyde overnight. The following day they were saturated in three steps with sucrose: the first two steps were either performed 2h at RT (Exp. IV, V, VI) or 8h at 4°C (Exp. VII, VIII) in 5% and 15% sucrose, followed by the third step: an overnight saturation with 30% sucrose (Exp. IV, V, VI, VIII) at RT (Exp. IV, V, VI) or 4°C (Exp. VII, VIII). Thereafter the TGs were frozen according to the same method as fresh frozen tissue. Tubes were stored at -80°C.

Sectioning and Fixation

Cryo-sections of 9μm (Exp. I-III) or 10μm (Exp. IV-VIII) were made with a Leica CM 1950 cryostat on pre-coated slides (coated with 3-aminopropyltriethoxysilane, Sigma) at -18°C. The sections were dried for 10-30min and fixed with 100% methanol (VWR) for 20min at -20°C (Exp. Ia), 3% paraformaldehyde (VWR) at RT for 10min (Exp. Ib, II) 1h (Exp. III) or 4% paraformaldehyde for 10min (Exp. IV, V, VI, VII, VIII). The sections were washed for 3x10min in PBS (without Ca+ and Mg2+). In Experiment IV during sectioning, every 10th slide, a DIFF-QUICK (Medion) staining was performed.

Lipid extraction

In Experiment IIb-VI and VIII a lipid extraction was performed either using 70% water-diluted ethanol (VWR) during one night (Exp. III, VI) or 15% ethanol (diluted in PBS) during 2 nights (Exp. IV, V, VIII). In Experiment I, IIa and VII no lipid extraction was performed.

Blocking and permeabilisation buffer step

Experiment Ib, Ila: the slides were washed for 3x5min in PBS and thereafter incubated in 0.2% Triton-X-100 (Sigma) for 2min, washed again 3x15min in PBS, incubated for 10min in 10% Negative Goat Serum (NGS, Gibco) and washed again 3x5min in PBS.

Experiment IIb-VIII: the slides were washed for 3x10min in PBS and thereafter treated with a blockingbuffer for 90min (Exp. III-VI) or 2h (Exp. VII, VIII) using Triton-X-100, Tween 20 (Sigma), NGS, (Cold Water) Fish Skin Gelatin (Sigma). An overview of the blocking and permeabilisation buffers used in each experiment is given in Tab 1.

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Tab. 1: Overview of the different blocking and permeabilisation buffers which were used in different experiments. Triton-X-100 (TX100), Tween 20 (T20), Negative Goat Serum (NGS), Fish skin gelatin (FSG). In experiment IIb the use of a blocking and permeabilisation buffer step during 90min was included for the first time. A = Fresh frozen, B = Pre-fixed. In Experiment V different blocking and permeabilisation buffers (a-e) were tested.
Primary Antibody incubation
Exp. I, Ila: The primary antibodies were diluted in PBS and incubated for 1h at 37°C.
Exp. IIb-VIII: After the use of the blocking and permeabilisation buffer the sections with the primary antibodies were incubated over one  (Exp. IIb-VIII) or three  (Exp. VII) night(s) at 4°C. The primary antibodies were diluted in the blocking and permeabilisation buffer.

Secondary Antibody incubation
Experiment I, Ila: First the slides were washed for 3x5min with PBS and thereafter incubated for 1h with the secondary antibodies at 37°C.
Experiment IIb-VIII: The slides were washed for 3x5min with PBS and thereafter incubated for 30min  (Exp. III-VIII) or 1h  (Exp.IIIb) with the secondary antibodies which were also diluted in the blocking and permeabilisation buffer. The slides were then washed again for 3x5min in white PBS and dipped twice in double distilled water.

Hoechst staining
Experiment I, II, IV-VIII: slides were washed 3x5min with white PBS, stained for 10min with Hoechst staining (Invitrogen, 1mg/ml, 33342) at a dilution of 1/200 and thereafter washed again 3x5min with white PBS.

Mounting
The slides were dried and mounted with cover glasses (VWR) and glycerin-DABCO (Exp. I, II, III) or Kaiser’s glycerol gelatin (Merck) (Exp. IV-VIII).

Imaging
Images were taken with an Olympus IX81 equipped with a Hamamatsu Orca (Hamamatsu Photonics) camera and the Cell* M analysis system (Olympus). Confocal microscopy was performed with a Zeiss LSM 710.

2.2. Cell cultures of porcine trigeminal ganglia
Trigeminal ganglia were excised from 3-week-old piglets after euthanasia with sodiumpentobarbital 20% (Kela). Ganglia were dissociated by enzymatic digestion with 0.2% collagenase A (Roche). The harvested cells were resuspended in culture medium (basic culture medium without glutamine and supplemented with nerve growth factor (30 ng/ml) (Sigma)) and seeded on collagen coated cover glasses. One day after seeding, cultures were washed with RPMI (Gibco) to remove non-adherent cells and from then on, culture medium was changed three times a week. After one week the cultured cells were washed with phosphate buffered saline (PBS) and fixed in 100% methanol for 20 min at -20 °C. All antibodies were diluted in PBS, all to a dilution of 1:100. Cells were incubated with each antibody for 1 h at 37 °C and all washes were performed with PBS.
2.3. Antibodies

Primary antibodies:

1. **Neurofilament 68: mouse-anti-NF68 Clone NR4 (Sigma)**
   
   NF is most abundant in neurons and consists of three major isoforms: NFL (68-70kDa) NFM (145-160kDa) and NFL (200-220kDa). The anti-NF68 antibody thus binds to the lightest subunit of NF (NFL) [23]. This antibody was raised in mice using NF68 from pig spinal cord. Reactivity has been shown with: pig, rat, chicken and human.

2. **Neurofilament 200: rabbit-anti-NF200 (Sigma)**
   
   NF200 antibodies stain the heaviest fraction of NF, which is most abundant in mature axons [23]. This antibody was raised in rabbit using NF200 from bovine spinal cord. Wide range reactivity is predicted.

3. **Neurofilament M: rabbit-anti-NFM (Enzo Life Sciences)**
   
   This antibody was raised in rabbit using purified primate NF proteins. Wide range reactivity is predicted.

4. **Neurofilament M: mouse-anti-NFM (Exp.VIII) (generated by B.Riederer, Lausanne, Switzerland) [21, 27, 35]**

5. **Alpha-tubulin: rabbit-anti-alpha-tubulin (Abcam)**
   
   The microtubule protofilaments are formed by alpha- and beta-tubulin dimers (see also introduction). Alpha-tubulin is present in neuronal as well as non-neuronal cells [1, 19]. This antibody was raised in rabbit using a synthetic peptide corresponding to C terminal amino acids 426-450 of human alpha-tubulin. Reactivity has been shown with: human, mouse, rat, chicken, Drosophila melanogaster, cow, gerbil, pig, Xenopus laevis, zebrafish, and was predicted to newt.

   
   Beta-III-tubulin is a neuron specific tubulin and is often used as marker for neuronal cells [1, 19, 24]. This antibody was raised in mouse using a peptide corresponding to the C-terminus of beta-III-tubulin. Cross-reaction with most mammalian species is predicted.

7. **Gamma-tubulin: rabbit-anti-gamma-tubulin (Abcam)**
   
   Forms the connection between MTs and the MTOC (see introduction). Gamma-tubulin is present in neuronal as well as non-neuronal cells [1, 40-41]. This antibody was raised in rabbit using a synthetic peptide corresponding to amino-acids 437-451 of Xenopus laevis gamma-tubulin. Reactivity has been shown with: human, mouse, rat, cat, pig, Xenopus laevis, zebrafish, and was predicted to: dog and Sea urchin.

8. **Myelin Basic Protein: rat-anti-MBP (Chemicon) [6, 36].**
   
   MBP is one of the major components of the myelin sheet of the central and peripheral nervous system [7, 29]. This antibody is was raised in rat using Bovine myelin basic protein and shows reactivity with human, bovine, sheep, rabbit, mouse, rat, guinea pig and chicken.

9. **P0 : rabbit-anti-P0 (generated by J.Archelos, Graz, Austria) [2, 12].**
   
   Myelin protein zero (MPZ or P0) is the most abundant protein in the myelin sheet of the peripheral nervous system and makes up 50-80% of the myelin proteins [13, 29]. This antibody was raised in mouse against recombinant rat P0-Extracellular Domain (P0-ED) [2]. Reactivity has been shown to: human, rat and mouse.

* kindly provided by Prof.N.Schaeren-Wiemers (Neurobiology Laboratory, Department of Biomedicine, University Hospital Basel, Switzerland)
10. **Tau:** mouse-anti-tau (generated by B.Riederer, Lausanne, Switzerland) [4, 33-34]. *A MAP mainly expressed in axons (see introduction) [10]. This antibody was raised in mouse against phosphocellulose-purified bovine MAPs [4].

11. **MAP1b:** mouse-anti-MAP1b (generated by B.Riederer, Lausanne, Switzerland) [8, 31-32]. *MAP1b is more abundant in the developing brain than in the adult brain (see introduction) [15]. Antibodies against recombinant MAP1B N-terminal fragments were produced in adult New Zealand White rabbits [5].

Secondary antibodies:

1. goat-anti-rabbit IgG Cy3 (Jackson Laboratories)
2. goat-anti-rabbit IgG Alexa 488 (Molecular Probes)
3. goat-anti-rabbit IgG FITC (Invitrogen)
4. goat-anti-rabbit IgG Texas Red (Invitrogen)
5. goat-anti-mouse IgG Cy3 (Milan Analytica)
6. goat-anti-mouse IgG Alexa 488 (Molecular Probes)
7. goat-anti-mouse IgG FITC (Invitrogen)
8. goat-anti-mouse IgG Texas Red (Invitrogen)
9. donkey-anti-rat IgG Cy2 (Jackson Laboratories)

*kindly provided by Prof. N.Schaeren-Wiemers (Neurobiology Laboratory, Department of Biomedicine, University Hospital Basel, Switzerland)
3. **Results**

3.1. **Immunofluorescence staining of porcine trigeminal ganglia**

3.1.1. **Experiment I**

Fresh frozen TGs tissue, was sectioned at –18°C, air-dried and fixed thereafter. One series was fixed in methanol (Exp.Ia) and another one in 3% PF (Exp.Ib). The same staining protocol was used as typically used for stainings of TG cultures (2min 0,2% TX100, 10min 10% NGS, 1h prim.Ab 37°C, 1h sec.Ab 37°C) [9]. Fluorescence microscopy of NF68 and beta-III-tubulin stainings revealed that the TG tissue sections did not contain TG cell bodies, and only transversally cut nerve fibers were visible (Fig. 6). Both fixation protocols led to a specific axonal immunofluorescence staining of NF68 (Fig. 6A and B, red). However, the immunofluorescence signal for beta-III-tubulin seemed less specific, since it was not confined to the axon (Fig. 6A and B, green). Although methanol fixation led to a much better immunofluorescence signal for NF68 (6A, red) than PF fixation (6B), based on the quality of the staining and the apparently less specific signal for the beta-III-tubulin staining, it was decided that PF fixation would be better suited for future experiments. PF fixation also appeared to maintain the structure of the tissue and cells much better.

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**Fig. 6**: Fluorescence microscopy of a fresh frozen TG show transversally sectioned axons. Sections were either fixed with methanol (A) or PF (B). Anti-NF 68 immunofluorescence signal (NF68, red, Texas Red (TR)) shows its location in axons. The anti-beta-III-tubulin staining (green, FITC) revealed beside axonal signals (yellow due to overlapping signal of NF68) also aspecific signals within the endoneural tissue, especially in the PF fixed tissue sections. (magnification: 200x)
3.1.2. Experiment II

In Exp. I, only transversally sectioned axons could be visualized. From Exp. II onwards, orientation of TG before sectioning was carefully assessed and sections were taken through the center of the ganglion, allowing observation of cell bodies. Based on experiment I it became evident that the use of the same staining method for TG tissue sections as for in vitro TG cultures was not sufficient to gain a satisfactory immunofluorescence staining pattern, we decided to establish an optimized protocol designed for tissue sections. We started with fresh frozen tissues and performed two different series of experiments. In Exp. IIa, (Fig. 7 and Fig. 8 A1, B1 and C1), we used the same staining method that we did in Exp. Ib (PF fixation), so we would be able to evaluate the staining on neuron cell bodies, which we missed during sectioning in Exp. I. In Exp. IIb (Fig. 7 and Fig. 8 A2, B2 and C2) we used a protocol which was described earlier in a publication where IF staining on sciatic nerves of mice was performed [36]. We did an overnight lipid extraction with 70% ethanol and worked with a blocking and permeabilisation buffer step of 90 min (0.2 TX100, 0.05% T20, 2.5% NGS). The antibodies were diluted in the blocking and permeabilisation buffer and primary antibodies were incubated over night at 4°C. Secondary antibodies were incubated for 1 h at 37°C. During evaluation of the staining there was a noticeable difference between the two methods. Using the blocking and permeabilisation buffer the antibodies did not only reach their target antigen better, but there was also much less aspecific binding (compare A2 with A1, B2 with B1 and C2 with C1 Fig. 7). In the NF68 staining one can see that the penetration of the antibodies is much better in Exp. IIb (Fig. 7 B2) than in Exp. IIa (Fig. 7 B1) and the alpha-tubulin staining shows that the blocking and permeabilisation buffer prevents background staining very well (Fig. 7 C2). We also did a Hoechst staining which shows that the neurons have lost their contact with the surrounding satellite cells during the handling procedure (Fig. 7 D). The black gaps between the cell bodies and the satellite cells illustrate this. Similar observations were made when sections were stained with beta-III-tubulin, NF200 and Hoechst (Fig. 8). In summary, the merged picture shows the overlapping immunofluorescence signal of NF68 and beta-III-tubulin in TG neurons (Fig. 7 A2) indicating that permeabilisation of TG tissue sections is necessary for sensitive and specific staining of intracellular components of the TG cells.
Fig. 7: Immunofluorescence localization of NF68 and alpha-tubulin in fresh frozen TG tissue sections. Cell bodies of neurons (c), satellite cells (s), axons or dendritic axons (a) are indicated. The usage of an overnight lipid extraction, a blocking and permeabilisation buffer step and an overnight incubation with the primary antibody (A2-D2) reduced not only the level of aspecific binding of the antibodies. But also clearly enhanced the specific binding of the antibody to its target. The reduction of aspecific binding can be seen best in the alpha-tubulin staining (green, FITC, C1&C2). The increase in sensitivity of the antibody binding is best illustrated in the NF68 staining (red, TR, B1&B2). Hoechst (blue, D1&D2) stains DNA and therefore cell nuclei. Since the DNA is more densely packed in satellite cells than in neurons, the cell nucleus of the neurons are much less visible. In the merged picture (top), the Hoechst staining shows that the neuronal cell bodies have shrunken within their cavities (arrows). (magnification: 200x)
Fig. 8: Immunofluorescence localization of NF200 and beta-III-tubulin in fresh frozen TG tissue sections. Cell bodies of neurons (c), satellite cells (s), axons or dendritic axons (a) are indicated. The usage of an overnight lipid extraction, a blocking and permeabilisation buffer step and an overnight incubation with the primary antibody (A2-D2) reduced not only the level of aspecific binding of the antibodies, but also clearly enhanced the specific binding of the antibody to its target. The reduction of aspecific binding can be seen best in the beta-III-tubulin staining (green, FITC, C1&C2). The increase in sensitivity of the antibody binding is best illustrated in the NF200 staining (red, TR, B1&B2). In the NF200 staining the axons appear in a tubular like structure, this compared to the NF68 (Fig. 7) staining. NF200 stains the “heavy chain” of NF which is more abundant in axons than in cell bodies (cell bodies are weaker stained for NF, compared to Fig. 7) and gives the axons this tubular appearance in a staining [23]. Hoechst (blue, D1&D2) stains DNA and therefore cell nuclei. Since the DNA is more densely packed in satellite cells than in neurons, the cell nucleus of the neurons are much less visible. In the merged picture (top), the Hoechst staining shows that the neuron cell bodies have shrunken within their cavities (arrows). (magnification: 200x)
3.1.3. Experiment III
Since the results of Exp.II were promising, a number of stainings were performed on a fresh ganglion (which was not stored, but sectioned directly after freezing) and using a selection of different antibodies. The use of the fresh ganglion was mostly an attempt to prevent the shrinking of the cell body observed in Exp.II. The blocking and permeabilisation buffer was also altered slightly: 0,1% TX100, 0,05% T20, 3% NGS. Unfortunately, the cell bodies had comparable to Exp.II shrunken. Further, certain antibodies gave a very nice staining with very little aspecific binding (NF200, Fig. 9 and NF68), whereas others gave some (NFM, Fig. 10) to a lot of aspecific binding (beta-III-tubulin, Fig. 11). A number of antibodies did not give a staining at all (MAP1b, tau).

Fig. 9: IF staining with NF200 (Alexa 488). Very little aspecific binding and a strong staining. Shrunken cell bodies are visible. (magnification: 200x)

Fig. 10: IF NFM staining with Cy3 (A) or Alexa 488 (B) as fluorescence molecule. The secondary antibody with Cy3 gives a stronger staining. Some aspecific binding (arrows) and shrunken cell bodies are visible. (magnification: 200x)

Fig. 11: IF Beta-III-tubulin staining (Alexa 488). A lot of aspecific binding is visible (beta-III-tubulin is neuron specific antibody and should not stain any non-neuronal cells, arrows). Shrunken cell bodies are visible. (magnification: 200x)
3.1.4. Experiment IV

Both in Exp.II and III cell bodies of the neurons collapsed. In these experiments, ganglia were frozen immediately after excision. In an attempt to preserve cell body collapse, a cryoprotecting method of fixation was applied prior to embedding. The ganglia were emerged in 4% PF over night at RT and thereafter saturated with sucrose in three steps (5%, 15% and 30%). The first two steps were performed for 2h and the saturation with 30% sucrose was carried out over night. The TGs were thereafter sectioned and fixed again for 10min with 4% PF. Also a lipid extraction was performed with 15% ethanol over two nights instead of 70% during one night, to see whether this would have an influence on the morphology of the tissue. The blocking and permeabilisation buffer used was a little less aggressive (0,05% TX100, 1% NGS, 2% FSG), since we tried to protect the tissue as much as possible. Most of the neurons indeed kept their round form and even subcellular structures such as the nucleoli are visible (Fig. 12 A2, Fig. 17). The beta-III-tubulin staining also revealed a net like structure within the cell bodies, probably showing the MT network within the cell (Fig. 12 A2). The NF68 and NFM staining were poor (Fig. 13 and Fig. 14), probably due to a lack of permeabilisation because of the lower TX100 percentage and/or a shorter duration of primary antibody incubation. The MAP1b and tau antibody did not stain at all. A P0 staining for peripheral myelin was also performed (Fig. 15), which was very successful. Also a control staining with a fresh frozen ganglion was made. The conditions of fixation, lipid extraction and permeabilisation (blocking and permeabilisation buffer step) were the same as for the pre-fixed ganglion. The observations made for the different antibodies were comparable to Exp.III. A very interesting fact was that the P0 staining did not work on the fresh frozen tissue at all (Fig. 16). It seems as if this component of the myelin, or at least the epitope recognized by the antibody, had been largely destroyed during the procedure. During sectioning every 10th slide a DIFF-QUICK staining was performed to get an immediate impression of the tissue structure. In Fig. 17 and Fig. 18 these stainings illustrate very well the difference between fresh frozen and pre-fixed tissue.

Fig. 12: IF beta-III-tubulin (green, Alexa 488, A2&B2) and Hoechst staining (blue, A1&B1) on pre-fixed TGs. The Hoechst staining illustrates that the neurons are surrounded by satellite cells and did not shrink during the freezing process. Even some subcellular structures such as the nucleolus are visible (arrow, A2). The MT network is visible as a netlike structure in the cell bodies. This is even more obvious by creating 1μm optical sections using confocal imaging (C1&C2). Since beta-III-tubulin is a neuron specific antibody it does not appear in the satellite cells (compare to Fig. 11, where non-neuronal cells are stained due to a lot of aspecific binding staining). (magnification A1-B2: 200x, C1: 200x, C2: 630x).
Fig. 13: NFM (green, Alexa 488) and Hoechst staining (blue) on pre-fixed TGs. Very weak and non-specific staining. In some places some shrinking of the neuronal cell bodies could still been seen (arrow). (magnification: 200x)

Fig. 14: NF68 (green, Alexa 488) and Hoechst staining (blue) on pre-fixed TGs. Very weak and non-specific staining. In some places some shrinking of the neuronal cell bodies could still been seen (arrow). (magnification: 200x)

Fig. 15: P0 (green, Alexa 488, B) and Hoechst (blue) staining on pre-fixed TGs. The nuclei of the satellite cells (s) illustrate the location of the neuronal cell bodies by surrounding them. The nuclei between the axons (Sw) are Schwann cells which myelinate the axons. (magnification: 200x).

Fig. 16: P0 (green, Alexa 488) and Hoechst (blue) staining of fresh frozen TGs. The neuronal cell bodies have stained aspecifically and the myelin antigen P0 epitope seems to have been largely destroyed. The nuclei of the Schwann cells which myelinate the axons are still visible (arrow). (magnification: 200x)
Fig. 17: DIFF-QUICK staining of thin sections of pre-fixed porcine TG. The neuronal cell bodies have much less shrunken (B, black arrows) in their cavities in comparison to fresh-frozen tissue (Fig. 18). Subcellular structures such as nucleoli are visible (white arrow) (magnification A: 100x, B: 400x).

Fig. 18: DIFF-QUICK staining of thin sections of fresh frozen porcine TG. Due to the lack of fixation prior to sectioning the neuronal cell bodies have shrunken in their cavities (arrows) and the nuclei have mostly been lost (compare to Fig. 17) (magnification: 100x).
3.1.5. Experiment V

Since some antibodies used in Exp.IV did not give a satisfactory staining (NF68, NFM) or did not stain at all (MAP1b, tau) different permeabilisation and blocking buffers were tested. The same ganglion as in Exp.IV was used. Since a lot of the neuronal cell bodies appeared shrunken and other structural aspects such as the myelin structure were lost, we could conclude that it is not advisable to freeze and reuse the tissue. The improvements between the different blocking and permeabilisation buffers concerning the staining quality of the tissue did not differ too much. Only the usage of more TX100 and NGS instead of FSG gave a noticeable difference (Fig. 20 and Fig. 22, comparing blocking and permeabilisation buffer b to e). The use of high concentration of NGS and TX100 would be advisable in the next experiment.

Fig. 19: NFM (red, Cy3), P0 (green, Alexa 488) and Hoechst (blue) staining on a pre-fixed TG. Blocking and permeabilisation buffer a: 0.05% TX100, 0.1% T20, 1% NGS, 2% FSG. The little amount of TX100 causes a very aspecific staining of NFM. (magnification: 200x)

Fig. 20: NFM (red, Cy3), P0 (green, Alexa 488) and Hoechst (blue) staining on a pre-fixed TG. Blocking and permeabilisation buffer b: 0.05% TX100, 0.1% T20, 3% NGS, no FSG added. A higher concentration of NGS resulted in much less aspecific binding and illustrates that FSG is probably not an ideal agent to prevent aspecific binding in this protocol. (magnification: 200x)

Fig. 21: NFM (red, Cy3), P0 (green, Alexa 488) and Hoechst (blue) staining on a pre-fixed TG. Blocking and permeabilisation buffer d: 0.25% TX100, 0.1% T20, 1% NGS, 2% FSG. The higher amount of TX100 gives a better permeabilisation and thereby a more specific staining. The low amount of NGS gives a bit of aspecific binding staining (staining of non-neuronal cells, arrow). (magnification: 200x)

Fig. 22: NFM (red, Cy3), P0 (green, Alexa 488) and Hoechst (blue) staining on a pre-fixed TG. Blocking and permeabilisation buffer e: 0.5% TX100, 0.1% T20, 1% NGS, 2% FSG. The higher amount of TX100 gives a better permeabilisation and thereby a more specific staining. The difference with 0.25% TX100 (Fig. 21) is slight. The low amount of NGS gives a bit of aspecific binding staining (staining of non-neuronal cells, arrow). (magnification: 200x)
3.1.6. Experiment VI

The best blocking and permeabilisation buffer (0.5% TX100, 1% NGS, 2% FSG) identified in Exp.V was now applied in a fresh pre-fixed ganglion. We still used FSG instead only NGS, which did not influence our staining too much. In Exp.VII we decided to only use high concentrations of NGS (see section 3.1.7). The sections were also treated with a 70% ethanol lipid extraction over night, which could explain the tissue not having such a nice structure as in Exp.IV (where 15% ethanol over two nights had been used). In the beta-III-tubulin staining, the net like appearance of the MT in the cell body, was even more visible than in Exp.IV (Fig. 23). Given that P0 does only stain for peripheral myelin (myelin produced by Schwann cells) we could locate on the staining for P0 the border between the peripheral and the CNS. This is illustrated in figure 27. Other stainings were still not very satisfactory. NFM did give quite some aspecific staining and MAP1b gave a weak staining which showed stained neuronal cell bodies and some highlights in the axons (Fig. 24). Tau on the other did again not give a staining at all. The antibody against Myelin Basic Protein (MBP), a molecule which does appear in the peripheral (PNS) as well as in the central nervous system (CNS), also yielded a weak staining with some highlighted spots (Fig. 25). These spots were places where the axons had been cut during sectioning of the tissue, and are therefore located at the surface of the section. The antibodies probably could access to the molecules more easily at these spots. A control staining with a fresh frozen ganglion was made. The conditions of fixation, lipid extraction and permeabilisation were the same as for the pre-fixed ganglion, with exception of the blocking and permeabilisation buffer (0.1% instead of 0.5% Triton-X-100). The observations made for the different antibodies were comparable to Exp.III.

Fig. 23: Beta-III-tubulin (green, Alexa 488, A2&B2) and Hoechst (blue) staining. The net like structure that the MTs produce are better visible with this protocol than in Exp.IV. In the picture A the nucleolus (arrow) of a neuron was captured. (magnification: 200x)
MAP1b (green, Alexa 488) and Hoechst (blue) staining. The cell bodies of the neurons as well as some spots in the axons are weakly stained. (magnification: 200x)

Fig. 24:

MBP (green, Alexa 488) and Hoechst (blue) staining. Only at places where the axons have been cut during sectioning the antibodies were able to create a staining (arrows). (magnification: 200x)

Fig. 25:

P0 (green, Alexa 488, B) and Hoechst (blue) staining. The different blocking and permeabilisation buffer did not have any influence on the staining of P0. (magnification: 200x)

Fig. 26:

P0 (green, Alexa 488) and Hoechst (blue) staining. The border between peripheral and CNS is very clearly visible (B). Schwann cells (Sw) myelinate the myelin sheet in the PNS. They possess an ellipse shaped nucleus in comparison to the oligodendrocytes (O) which myelinate the CNS and have a more round nucleus. Fewer nuclei are visible in the CNS in comparison to the PNS. This is due to the ability of the oligodendrocytes to create several (up to 50) myelin sheets, in comparison to the Schwann cell, which rolls itself around the axon and thereby only creates one myelin sheet [26] (magnification: 200x).

Fig. 27:
3.1.7. Experiment VII

Since some of the antibodies produced only a weak staining or no staining at all in Exp.VI, the procedure was repeated and the primary antibodies were incubated for 3 nights instead of one night. The blocking and permeabilisation buffer was applied during 2h instead of 90min to enhance permeabilisation. Very high concentrations of TX100 and NGS were used (0.5% TX100 and 10% NGS) to prevent any aspecific staining, which is likely to occur during incubating over such a long period. There was no lipid extraction step used, to see whether the tissue would be less harmed in this way. The NF68 staining indeed improved a lot (Fig. 28). The tau and MAP1b staining were still very inconclusive. All other antibodies (NF200, beta-III-tubulin, alpha-tubulin and gamma-tubulin) gave such a strong staining that aspecific binding and actual staining could not be differentiated anymore. There was little difference seen in tissue structure with Exp.VI, where a lipid extraction step had been applied.

![NF68](image)

**Fig. 28:** IF NF68 (red, TR) and Hoechst (blue) staining. NF68 did not give any staining when incubated during one night. Staining was achieved when primary antibodies were incubated during 3 nights. This shows that IF staining and incubation protocols can differ according to the antibody used. (magnification: 200x)
3.1.8. Experiment VIII
In this last trial all the conclusions gathered in the previous seven experiments were assembled. The ganglion was first incubated for 2 days in 4% PF and then saturated with sucrose during 2.5 days in three steps (5%, 15% and 30%). After sectioning a short fixation (4% PF, 10min) was applied followed by a lipid extraction incubating the sections for 2 nights in 15% ethanol. The staining was performed after a 2h permeabilisation with the blocking and permeabilisation buffer (10% NGS and 0,5% TX100). The primary antibodies were incubated during one night and were dissolved in the blocking and permeabilisation buffer. The tissue structure had been conserved very well and most stainings (NF200, NFM, beta-III-tubulin and alpha-tubulin) were successful (Fig. 29, Fig. 30, Fig. 31 and Fig. 32). The gamma-tubulin staining which was applied here for the first time within the standard protocol did not give a staining. As shown in Exp.VI and VII, NF68 antibodies need to be incubated for 3 nights to achieve a relevant staining. As a result, no staining of NF68 has been observed in Exp.VIII.

![Beta-III-tubulin](image1)
![Alpha-tubulin](image2)

**Fig. 29:** IF beta-III-tubulin (green, FITC) and Hoechst (blue) staining. The tissue structure is preserved very well, due to extensive fixation treatment prior to sectioning. (magnification: 200x)

![NFM](image3)
![NF200](image4)

**Fig. 30:** IF alpha-tubulin (green, FITC) and Hoechst (blue) staining. (magnification: 200x)

**Fig. 31:** IF NFM (red, TR) and Hoechst (blue) staining. Due to excellent preservation even the NF net like structure within the cell body is visible (arrow). (magnification: 200x)

**Fig. 32:** IF NF200 (red, TR) and Hoechst (blue) staining. (magnification: 200x)
3.2. Cell cultures of porcine trigeminal ganglia

To see whether porcine TG cell cultures are representative for the structures observed in vivo situation the same stainings are performed on 8 day old porcine TG cell cultures. The structure and organization of NF and the microtubules does not differ from observations made with sections (Fig. 33 and Fig. 34). Whether the MAP1b and tau staining did or did not give a staining was not possible to detect with an IF microscope. Using the confocal microscope and analyzing the emitting wavelengths, detecting a significant staining was possible (Fig. 37). The staining of MAP1b and tau were very faint, probably due to the low occurrence of these molecules (Fig. 35 and Fig. 36). The gamma-tubulin staining was also analyzed with the confocal microscope and MTOCs could be appointed to almost every non-neuronal cell (Fig. 38). It was not possible to connect a MTOC to the neuronal cells present in the culture.

![Images](image1.png)

**Fig. 33:** Immunofluorescence localization of beta-III-tubulin (green, FITC, B1&B2), NF200 (red, TR, C1&C2) and Hoechst (blue) staining of porcine TG cultures. The net-like structure of MTs and NF cannot be seen due to the limited imaging technique and the thickness of the culture. They can be seen using confocal microscopy (Fig. 34). (magnification: 200x)
Fig. 34: Immunofluorescence localization of beta-III-tubulin (green, FITC, C), NF200 (red, TR, B) and Hoechst (blue, A) staining of porcine TG cell culture. Due to confocal imaging the net-like structure of the microtubules, which can also be observed in IF staining of TG sections, can be seen (C, arrow). (magnification: 200x)

Fig. 35: Immunofluorescence localization of MAP1b (green, FITC, C), NF200 (red, TR, B) and Hoechst (blue, A) staining of porcine TG cell culture. Significant staining can be seen in neurites (arrows). By performing a spectrum analysis of the emitting waves, it was possible to prove that the signal’s source was not an overlapping of the TR signal. The same analysis was done for tau, an illustration of this is given in Fig.37. (magnification: 630x)
Fig. 36: Immunofluorescence localization of tau (green, FITC, C), NF200 (red, TR, B) and Hoechst (blue, A) staining of porcine TG cell culture. Significant staining can be seen in neurites. By performing a spectrum analysis of the emitting waves, it was possible to prove that the signal’s source was not an overlapping of the TR signal. This is illustrated in Fig. 37. (magnification: 630x)

Fig. 37: Spectrum analysis of an immunofluorescence localization of tau (green, FITC), NF200 (red, TR) staining of porcine TG cell culture. The peak between ~500-540nm (between the two lines) proves that there is a significant FITC signal. The peak between ~560-650nm represents the TR emission signal. (magnification: 630x)
Fig. 38: Immunofluorescence localization of gamma-tubulin (green, FITC, C), NF68 (red, TR, B) and Hoechst (blue, A) staining of porcine TG cell culture. The MTOCs are visible as little green spots (C and D) and are always adjacent to a nucleus. During optical sectioning it was not possible to prove a significant correlation between a MTOC and the neuronal cell nucleus (n). The MTOCs close to the nucleus (thin arrows) of the neuronal cell were all correlated to non-neuronal cells lying on top of it. (magnification: 630x)
4. **Discussion**

4.1. **Best Protocol for Immunofluorescence staining of porcine trigeminal ganglia**

A great staining is a combination of different elements. First, the tissue needs to be preserved as much as possible. A tissue that is too damaged can leave too much space for doubt whether the staining result is relevant or not. This was shown in Exp.IV where the staining for P0 was negative in fresh frozen tissue (Fig. 16) but gave a very nice staining in pre-fixed tissue (Fig. 15). A second issue that needs consideration is a combination of good permeabilisation and blocking of aspecific binding. This was illustrated in Exp.V where we tested different blocking and permeabilisation buffers (Fig. 19 to Fig. 22). Then there is also an individual difference according to the primary antibody used. The incubation time, of the primary antibody, needed to obtain a nice staining result differed according to the antibody which was used. P0, alpha- and beta-III-tubulin did give a very nice staining after one night staining on pre-fixed tissue. The NF68 did need 3 nights of incubation to give a powerful staining. It is therefore advisable that one tests different periods of incubation before concluding that an antibody does not give a good staining after just one night of incubation. The tau antibody did not give a staining at all. It was first thought that the fact that it was developed from human tissue, could have been the reason for the antibodies not recognizing their target. But since a staining was obtained on TG cell cultures a low occurrence of this molecule, the destruction of it or it’s epitope during the staining protocol seem more reasonable. The same accounts for the MAP1b antibody, which did give a very inconclusive staining in Exp.IV. The gamma-tubulin antibody did give a staining on cell cultures but not on sections. This and the MAP1b and tau stainings will be further discussed in section 4.2. A fixation method which was not tried, and which is known for its excellent tissue preservation is paraffin embedding. The main reason that paraffin was not used here is that paraffin embedding is known for causing problems with antibody penetration [28]. If one wishes an even better tissue structure, this could be a method which could be undertaken.

To conclude a summary of the most ideal method to do an IF staining on porcine TGs:

**Excision and Pre-fixation**

Euthanisation of pigs with sodiumpentobarbital 20% and immediate excision of TGs.

Pre-fixation of TG with 4% PF during 2 nights at 4°C.

Saturation with sucrose, performed in three steps, all at 4°C. First two steps (5% and 15%) at least during 8h. The last saturation step is performed over night or until the ganglia have disseminated to the bottom of the vessel.

**Freezing**

TGs are submerged in a freezing compound. The tube containing the TG and the freezing compound are thereafter submerged in dry-ice cooled isopropanol and frozen at exactly -40°C.

TGs can be stored at -80°C.

**Sectioning and Fixation**

Cryo-sections of 10μm are made and dried during 10-30min. Thereafter a fixation with 4% PF is performed.

The sections are washed for 3x10min in PBS.

To control the tissue structure a DIFF-QUICK staining can be performed.

**Lipid extraction**

The sections are incubated in 15% ethanol during two nights.

**Blocking and permeabilisation buffer step**

The sections are washed for 3x10min in PBS. Then they are incubated during 2h in a blocking and permeabilisation buffer containing 0,5% TX100 and 10% NGS.
Primary Antibodies
The sections with the primary antibodies are incubated over one night at 4°C. This period can be adapted according to the antibody (see previous discussion). The primary antibodies were diluted in the blocking and permeabilisation buffer.

Secondary Antibodies
The slides are washed for 3x5min with PBS and thereafter incubated for 30min with the secondary antibodies, which are also diluted in the blocking and permeabilisation buffer. The slides where then washed again for 3x5min in white PBS and dipped twice in double distilled water.

Hoechst staining
Slides are washed 3x5min with PBS, stained 10min with Hoechst staining at a dilution of 1/200 and thereafter washed again 3x5min with PBS.

Mounting
The slides are dried and mounted with cover glasses and a fluorescence protecting agent.

4.2. Comparison of IF staining on thin sections versus on in vitro cell cultures of porcine trigeminal ganglia
IF stained TG cell cultures and thin sections of TG were analyzed to see whether they are somehow comparable or not. Since cell cultures are widely used, not only in herpesvirus research, but also in about any other microbiological and cell biological research field, it is of main interest to know whether findings made in vitro can be extrapolated to the in vivo situation. From a microscopical point of view, the structure of TG neurons in culture probably does not represent the PUNs known in vivo. The cell body, for example, does not lie in the center of the cell. In a cell culture the polarity which is present in a living organism is missing (PNS and CZS), and it does therefore not astonish that instead of an axon and a dendritic axon a variety of branches is formed. Since these neurons are forced into a new environment they are highly active cells, trying to sustain in a less ideal milieu. To maintain their growth and different structure these neurons have to express different genes than TG neurons embedded in a ganglion. When it comes to the MT transport system and NF the same net-like structure as in sections could be observed in cell cultures. A very interesting approach would be to see whether the same transport molecules (dynein and kinesin types) are present in cell cultures compared to in vivo tissue. In contrast to thin sections, confocal microscopy for cell cultures revealed very little, but significant staining of MAP1b and tau. One possible explanation for this observation may be that MAP1b and tau are probably molecules with low abundance and were probably therefore missed (tau) or gave inconclusive results (MAP1b) using IF microscopy on thin sections (see Exp.VI). Another possibility is that, since cultured neuronal cells are highly active, the expression of MAP1b and tau is higher in cell cultures than in vivo, therefore allowing detection by confocal microscopy. No P0 and MBP staining was accomplished, which was different from the thin sections and is probably due to the fact that no myelinating Schwann cells or oligodendrocytes were present in the culture. Myelin-forming cells need special medium and growth factors, such as forskolin, pituitary extract, N2 supplement and ascorbic acid to be active [20], which were not present in our cultures [9]. As mentioned in section 4.1 the gamma-tubulin staining did work in cell cultures, but did not in thin tissue sections. Further, in TG cell cultures every non-neuronal cell nucleus could be co-localized with a MTOC. This in comparison to neuronal cells, where no MTOC was found adjacent to the nucleus. Since only two neuronal cells could be evaluated, these findings need further investigation and repetition of these experiments. One reason, for cultures cells displaying MTOCs and thins sections not, could be that cultured cells are highly active and maybe gamma-tubulin is more abundant due to the high MT activity. It can be concluded that for certain structures cell cultures are probably representative
for an in vivo situation. But for situation where polarity and differentiation is involved, interpretations have to be made carefully.

4.3. Conclusion
These experiments were initiated, on the one hand, to establish a reliable protocol for IF staining of porcine TG, but on the other hand also with the objective to take a closer look at the MT organization in TG neurons. IF staining sections with alpha- and beta-III-tubulin has shown that the MT transport system in neurons can be visualized, but only to a limited amount, which does not allow any interpretation concerning the detailed structure of the MTs. A net-like structure can indeed be seen, but it is impossible for example to see how MTs are divided at the point where dendritic axon and axon of the PUN divide. This may also mainly be due to the fact that this dividing point is rarely ever seen on a section and even if, the resolution of an immune-fluorescence microscope or confocal microscope (200nm) is too limited. Since this dividing point cannot (easily) be found on thin sections for fluorescence microscopy, electron microscopy may also be unable to provide an answer, seen the need for even thinner ultrathin sections for this approach. To narrow down onto the differences between MT transport molecules within the dendritic axon and the axon of PUNs mass-spectometry on CNS-directed axons and periphery-directed dendritic axons could be a possibility. Very discrete differences in molecule composition could be detected and would give a clearer picture than IF staining can ever provide.
5. References


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Acknowledgements
This was the first project for me to work in a laboratory and I very much enjoyed this experience. Since everything was new to me, I needed a lot of help from the start to the very end. It was therefore great to be surrounded by so many helping hands. I want to thank my promoter Prof. Dr. Herman Favoreel who is always open for scientific “brain-storming” and is an example for excellent leadership. A big thank you goes to Nina for being a great co-promoter by not only showing me the way in the lab, but also being such a sunny person. Further I want to thank Nick for giving me good start up in de lab and providing me with a great thesis of his. Without Natasha I would have probably lost my head more than once and I am very thankful for her guiding me whenever I required it. A big thank you goes to all the other people in the lab who have helped me out with either knowledge, material or just good conversations: Marc, Lennert, Korneel, Uladzimir, Matthias, Ahmed and Ut. I also want to thank Philippe for making the light microscope pictures of the DIFF-QUICK stainings. The bottle of “Appenzeller” is on its way! Another fellow student which has helped me a lot was Joachim and I want to thank him for sharing his knowledge with me and the scientific conversations we have, which are always a lot of fun. Special thanks go to Prof. Dr. Nicole Schaeren-Wiemers (Neurobiology Laboratory, Department of Biomedicine, University Hospital Basel, Switzerland) and her lab for providing me with antibody samples and a lot of experience. I especially want to thank Beat Erne for his expertise in IF staining and confocal microscopy. I am also very grateful for the (sometimes last minute) help by phone I got from Thomas Zeis and Jochen Kinter. Last but not least I want to thank Bart and my family for all the love and support they have given me over the last years.