0000000185 – DynaRibo

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Dynamics of ribosome local supply in axon regrowth

DynaRibo

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1. EXCELLENCE

1.1. Pre-proposal's context, positioning and objectives

Background, hypothesis and state-of-the-art

Axon biology in neurodevelopment and regeneration

Neuronal cells are highly polarized and organized into structurally and functionally distinct sub-domains, including the soma, dendrites and the axon, which is responsible to emit electrical signals to target cells. Elucidating the mechanisms of axon formation stands as a cornerstone to further understand the development, maturation and plasticity of neural circuits during development. This requires multiscale approaches to link the axon structural and regulatory properties (cytoskeleton architecture, axonal transport, subcellular compartmentalization) to its functions.

Importantly, deciphering axon formation has a major impact to address neuronal circuit repair. In adult mammals, neurons of the central nervous system (CNS) fail to spontaneously regrow their axon after injury (unlike in the peripheral nervous system (PNS) or embryonic neurons), hindering functional reconnection. To address the axon regeneration failure of neurons in the injured CNS, the field has looked at multiple aspects encompassing extrinsic factors (*1*, *2*) and intrinsic properties of neurons themselves (*3*), including regulation of gene expression evaluated in the soma. Yet, CNS axon regrowth remains an unmet need. In the Schaeffer team, our projects aim to go beyond this knowledge by exploring how gene expression is regulated locally in the axon.

The axon regrowth capacity may be limited by ribosome axonal supply

It is now established that the axon behaves autonomously from the soma, with its own gene regulatory programs. For this it relies on local protein synthesis from mRNAs that are transported, stored and recruited for translation locally. Axonal translation of specific mRNAs is key for many steps of circuit formation and maturation (*4*), and for circuit function (*5*). This decoupling between soma and axon gene regulatory programs is, at least in part, driven by the compartmentalization of organelles (mitochondria, endosomes, tubular ER) that act as local protein synthesis platforms (*6*).

Ribosomes are the functional units of protein synthesis. Since they are required for local protein synthesis in the axonal compartment, a key question is how their local distribution relates to axon maintenance and regrowth. Early studies linked axonal ribosome amounts to regrowth capacity, based on observations that adult CNS growth cones contain less ribosomal proteins than PNS and embryonic CNS (*7*). Besides, local translation is limited by the translation initiation step, which is directly impacted by ribosome number (*8*).

Together, these observations support our global hypothesis that ribosome supply in the axon is a limiting step of local protein synthesis required for regrowth.

However, several questions remain unresolved:

How are ribosomes locally supplied within axons? What is the contribution of axonal active transport versus passive diffusion of ribosomal subunits? What are the mechanisms that control ribosome distribution and mobility in axons? Finally, how do ribosome movements within axons relate to their regrowth capacity?

To address these questions, we need a dynamic visualization of ribosomes and protein synthesis events in axons, and a quantitative assessment of ribosome local supply in relation to the regrowth capacity. Yet, the detection of ribosomes in distal neuronal compartments has long been challenging. Recent advances in imaging have confirmed local presence and functionality of ribosomes in axons of the mammalian brain (*9–11*). A real-time imaging approach is now required to capture ribosome movements in live axons, which will provide key insight into how ribosome local supply in axons relate to their regrowth capacity.

Objective and experimental aims

The overarching objective of the project is to provide single-molecule real-time imaging and quantitative analysis of ribosome movements in regrowing axons.

It is divided into four aims:

1) optimization of high-resolution imaging of ribosomes in regrowing axons;
2) development of an analysis pipeline for automated detection and trajectory tracking;
3) correlative measurements of ribosome movements in relation to other organelles in axons;
4) data-driven modelling of axon regrowth capacity based on ribosome axonal supply.

Preliminary results and general methodology

Protein synthesis occurs locally in regrowing axons

Until now, studies of CNS axon regrowth have mainly relied on soma-focused profiling methods (such as single-cell transcriptomics), thus missing the compartmentalized aspect of translation regulation specifically in the axon. Previously, we optimized an *ex vivo* method of retina ganglion cell (RGC) regrowth based on mouse retina explant culture (*13*). This model recapitulates CNS growth and regrowth with high fidelity and gives access to the axonal compartment. Based on the detection of O-propargyl-puromycin (OPP) incorporation into newly synthesized peptides, we found that new protein synthesis occurs locally in regrowing axons: not only in the growth cone (the leading structure at the axonal tip whose formation is limiting for the regrowth process) but also along the axon shaft, distributed as discrete hotspots (***Figure 1***). These preliminary data suggest that protein synthesis distribution within the axon compartment is an essential aspect of regrowth.

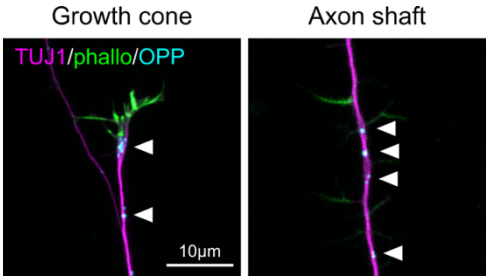


Figure 1 Confocal images of RGC axons regrowing from a post-natal retina explant, showing new protein synthesis (OPP labeling) in the growth cone and in the axon shaft. The microtubules are labeled in magenta (βIII-tubulin, TUJ1 staining) and the actin in green (phalloidin staining).

Development of a tool to image endogenous ribosomes and protein synthesis events

Detecting ribosomes in distal neuronal compartments has long been challenging, but recent imaging advances have confirmed their presence in axons of the mammalian brain, using antibodies on fixed tissue (*9–11*). Yet, a dynamic visualization of ribosome movements in live axons is incompatible with antibody labeling, as antibodies cannot cross the cell membrane. We have recently developed an original approach to detect both ribosomes and protein synthesis events in axons in real-time.

General experimental strategy and feasibility

In this project, we will leverage mouse retina explant cultures as a faithful *ex vivo* model of RGC axon regrowth (*13*) to image ribosomes and protein synthesis in regrowing axons in multiple conditions of interest: a high regrowth capacity in post-natal explants, and a promoted regrowth capacity based on experimental deletion of the mTOR inhibitor Pten, which despite limited efficacy and side effects, remains the best paradigm of adult regrowth (*17*). This will be obtained by intravitreal injection of AAV2-Cre in eyes of adult Pten-floxed mice, as AAV2 have a high tropism for RGCs. Imaging will be done using single-molecule sensitive microscopes based on total internal reflection fluorescence (TIRF), in collaboration with Pierre Mangeol (IBDM), an expert in single-molecule and super-resolution microscopy (*18*). TIRF microscopy is adapted to thin structures such as axons because it enables excitation of fluorophores located close to the coverlip (<200nm), thus limiting the signal-to-noise ratio. This approach is adapted to detect single molecules and will considerably improve the detection and tracking methods.

Using retina explant cultures, we have already conducted a pilot study to track endogenous organelles (lysosomes) using a dual approach: algorithm-based tracking (nearest neighbor) and deep learning using synthetic data (***Figure***). We will further improve these strategies for ribosome tracking and compare them with an automated kymograph analysis tool previously developed (*19*). We will extract quantitative information to uncover key parameters of ribosome local supply during axon regrowth, including: speed, directionality, active/passive transitions, subcellular distribution, and correlations with movements of other organelles enabled by simultaneous multi-object tracking in axons. This will lay the groundwork for elucidating ribosome transport mechanisms during axon regrowth.

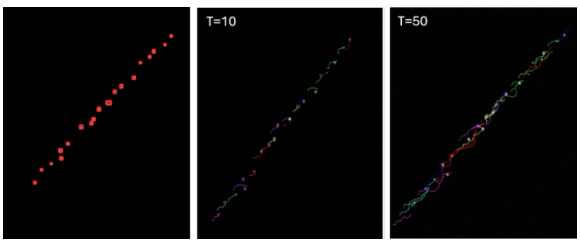


Figure 2 Example of particle detection using YOLOv8 and tracking over multiple timepoints using DeepSort on synthetic data.

Sex bias consideration in the experimental design

Our preliminary data show no significant difference in axon regrowth of explants originating from males and females. Yet, some studies point to sex-dependent gene expression variations in nervous system regeneration (*20*). So in this project, we will pay attention to any sex bias and ensure an equivalent representation of males and females across samples.

Originality and novelty

The project relies on both conceptual and methodological original aspects:

- First, the development of a widely-usable imaging tool for single-molecule real-time tracking of endogenous ribosomes;
- Second, our unique methodology that combines a CNS axon regrowth model with computer vision-assisted tracking of intra-axonal transport;
- Third, the currently unaddressed question of ribosome axonal supply and, as a follow-up, the underlying mechanisms of ribosome intracellular transport. By addressing this question, the proposed project will take a major step in understanding how gene expression is locally regulated in axons, using CNS axon regrowth as a specific phenotypic readout.

For the first time, this project will leverage an interdisciplinary approach to gain insight into ribosome distribution and dynamics in the axonal compartment. Ultimately, the use of a faithful model of CNS axon regrowth will enable a transition from *ex vivo* to *in vivo* with highly transposable results.

1.2. Interdisciplinary dimension of the project

By integrating cutting-edge single-molecule imaging of endogenous ribosomes with computer-vision–based tracking, this project is intrinsically interdisciplinary, by bridging neurobiology, optical imaging, and computational analysis – complementary expertises of the two host teams.

The project requires technical and conceptual expertise in neurobiology (expertise of Julia Schaeffer (*13*, *21*, *22*)) notably for the set-up of retina explant cultures, and advanced imaging (expertise of our collaborator Pierre Mangeol (*18*)) for optimization of high-resolution real-time imaging within axons. The project also includes a fundamental cell biology dimension to elucidate how protein synthesis is compartmentalized within axons, a key process in axon specification, maintenance and stress response. The project requires to develop technical skills and conceptual knowledge of neurobiology:

- set-up of the faithful model of retina explant cultures, which enables to focus on the axonal compartment itself to capture intra-axonal mechanisms associated to local protein synthesis;
- high-resolution and real-time imaging of subaxonal dynamics of biological objects;
- analysis of ribosome density and mobility in relation to the regrowth capacity;
- analysis of dynamics of ribosome local supply in the axon in relation to other organelles.

Computer vision (expertise of Severine Dubuisson (*23–25*)) enables to track movements of biological organelles with high precision, efficiently handle large datasets and automate human error-prone processes. This discipline has a major added value to the project because it enables real-time analysis of ribosome dynamics in regrowing axons, ensuring consistent and objective measurements in the context of an original biological question currently unaddressed. By tracking individual ribosome trajectories, we will be able to extract quantitative information that will provide us with key parameters of ribosome local supply in axon regrowth. Additionally, in the long term, deep learning may improve the accuracy and adaptability of these tracking systems. So computer vision is an integral part of our methodology to test the project hypothesis:

- test and validation of a custom algorithm based on real-life data;
- automation of ribosome tracking that would be difficult to achieve manually;
- possibility to track multiple objects simultaneously, a critical aspect to link ribosome transport to other organelles in regrowing axons and set the basis to decipher the underlying transport mechanisms.

Our original interdisciplinary approach goes beyond the current state-of-the-art (annotation of kymographs for example) that requires a lot of manual curations. Here, we will take advantage of high-throughput imaging in microfluidic chambers to serve different purposes: optimize a new tool widely-usable; develop a cost-effective and highly precise pipeline of particle tracking; and answer a biological question about how the local supply of ribosomes relates to the regrowth capacity. Results of this project will set the basis for long-term objectives to elucidate and manipulate ribosome transport mechanisms in order to unlock axon regrowth after injury. In this project, mathematical modeling of ribosome local supply will provide a powerful framework for understanding the complex dynamics and regulation of this process in the context of axon injury and regeneration. In the long term, it will be extremely valuable to explore the mechanisms

underlying ribosome movement and storage in axons, to predict how external cues impact it, and to evaluate potential therapeutic interventions to improve axon regrowth and/or limit axon degeneration in multiple neurological contexts.

The PhD project will be hosted at IBDM in Julia Schaeffer's team. Data acquisition will be performed on the optical imaging facility of IBDM, in collaboration with a post-doc in the Schaeffer team expert in high-resolution imaging. Weekly one-to-one meetings with Julia Schaeffer and Severine Dubuisson will be dedicated to project advancement. Severine Dubuisson will also meet the student frequently to guide him/her for the development of the tracking method. The student will participate in lab meetings of both teams and have the opportunity to present his/her research at internal seminars at IBDM, LIS and CENTURI (on the basis of one seminar per year).

The student will also have a privileged contact with Tafalgie Therapeutics, a spin-off of the CNRS whose research focuses on neuropathic pain. The link between the host institute IBDM and Tafalgie Therapeutics is very strong, and the thematics pursued by Tafalgie Therapeutics is highly relevant to the project's aims – to gain insight into fundamental axon biology and its regulation in a pathological context. In this regard, the student will benefit from the mentorship of Aziz Moqrich, CSO of Tafalgie Therapeutics in order to discover scientific research under the prism of innovation business and pre-clinical/clinical trials. This will take the form of regular meetings with Aziz Moqrich and short secondments with various members of the team to discover Tafalgie research activities and functioning, as well as communication of his/her own results to bridge fundamental mechanisms of axon biology and principles of pathophysiology of pain.

Throughout the PhD training, we will provide mentorship to the candidate to accompany his/her career development. One-to-one meetings will be dedicated to personal development, including skill enhancement and career expectations. We will mentor the student for post-doctoral grant applications (if applicable) and for interview processes, whether within or outside academia. More generally, in our team, we foster a dynamic and friendly scientific atmosphere and take into account each and everyone's differences. Our idea is to breed inclusion of all team members, make them feel that they belong to a group and provide them with the taste for research and science.

2. IMPACT

2.1. Expected impact of the project on the candidate's career

The proposed PhD research project and training will have a significant impact on the future career prospects of the PhD candidate, whether within academia or beyond. Through the completion of the project, he/she will acquire a set of advanced skills and knowledge that will enhance his/her expertise and open up diverse career opportunities.

First, the project will allow the PhD candidate to develop a deep expertise in a specific subject across technical, methodological, and conceptual levels. The student will receive training of cutting-edge approaches in neurobiology, imaging and computer vision. Addressing the proposed original research question will involve the development and optimization of techniques and the acquisition of advanced analytical skills on a complex, multimodal question. The project and training will also allow the PhD candidate to push the borders of current conceptions in the axon regeneration field, by shifting from a "soma-focused" approach to viewing the axon as an autonomous compartment, fostering innovative and critical thinking. The candidate will also develop his/her creativity through the development of imaging and analysis tools and their potential adaptation to a variety of biological questions. Depending on the project advancement, the candidate will be offered to explore side questions, for example how ribosome distribution is remodeled in axons encountering an external cue. Altogether, the project and training will provide the student with a comprehensive research experience. They will make him/her highly competitive both for postdoctoral positions as well as for positions in private, non-academic sectors where expertise is highly valued for its ability to solve complex, real-world problems.

Second, the interdisciplinary aspect of the project will strongly encourage the candidate to think out of the box. Our original research question and methodology bridges multiple fields – local protein synthesis, axon regeneration – and is inspired by different approaches – particle tracking, mathematical modeling. Being in touch with multiple ways to work and think, the candidate will develop different yet complementary skills and knowledge and widen his/her set of competencies. The methodological design of the project will also provide the candidate with a complete picture of a biological question, from data generation to analysis and interpretation.

Third, the PhD experience will give the candidate multiple professional intersectoral skills:

- **Project management and leadership skills**: the candidate will be encouraged to gain autonomy on the project. He/she will also be given the opportunity to supervise an intern from year 2.
- **Presentation, communication and dissemination**: we will give the candidate the opportunity to participate in conferences to present his/her methods and results to reach experts in the fields, as well as the general public through participation in festivals of popular science. The student will have the opportunity to showcase his/her own data towards a communication purpose. Finally, we will guide the PhD candidate through assembly of his/her results towards publication in a high impact factor journal.
- **Collaboration and networking**: the project we propose has the advantage of being co-supervised by two experienced researchers with strong networks in their respective communities in Marseille, in France and internationally. Notably, the PhD student will benefit from the Turing Centre for Living Systems (CENTURI) network, a dynamic interdisciplinary consortium of Marseille into which IBDM and LIS are embedded. Interactions with CENTURI will provide collaborative opportunities as well as participation in scientific events such as the yearly Hackathon. In addition, through the international partnership we propose, the candidate will perform an internship in the Geometric Intelligence Lab led by Nina Miolane in UC Santa Barbara, in order to open perspectives on biological imaging analysis.
- **Introduction to intersectoral research activities**: we propose an intersectorial partnership with Tafalgie Therapeutics, a spin-off of the CNRS dedicated to research on neuropathic pain. The student will have regular contacts with Aziz Moqrich, CSO of Tafalgie Therapeutics, and various members of the team, to discover the innovation and clinical application in the environment of a biotechnology start-up.

In conclusion, the proposed PhD research and training project will give the candidate a robust set of competencies, including advanced technical skills, critical thinking, and communication abilities, which will enhance his/her career prospects across a wide range of professional environments.

2.2. Expected impact for the thematic axis

By developing an unprecedented method to image ribosome dynamics at single-molecule level and in real-time, this project will significantly advance our understanding of biological regulation within axons. Importantly, our novel ribosome-tracking tool and tracking methods can be applied across diverse cell types, developmental stages and disease contexts, opening the possibility to study what controls intracellular ribosome dynamics and how it responds to stress or pathology. Sharing our concepts and techniques will foster collaborations in advanced nanoscale imaging of compartmentalized protein synthesis. For example in cancer research, we will collaborate with Fanny Mann's team at IBDM to decipher how ribosome dynamics in the axon contributes to tumor innervation by adult neurons.

By investigating the cellular and molecular mechanisms underlying axon injury and regeneration, this project addresses both the scientific and societal challenge of repairing the injured CNS. As life expectancy rises and behavioral changes occur, the global prevalence of CNS disorders has surged. Unfortunately, there is still no effective treatment to prevent the debilitating consequences of these conditions, making CNS repair one of the most pressing health issues of the 21st century.

In my research team, we are bridging different areas by exploring local regulation of gene expression in the axon as a signature of its regrowth capacity upon injury. In the field of CNS repair, our mechanistic approach looking at the subcellular level of axon regrowth will provide a novel perspective on the molecular and cellular processes in injured axons, an underexplored aspect of axon regeneration. In the field of local translation, our focus on CNS injury and repair adds to the current international research that focuses on the injured PNS or on synapse formation and activity in the intact CNS. So results of this project will advance our knowledge of what makes an injured axon able (or rather unable) to regrow and reconnect in the context of CNS repair. They will provide an axon-specific view of how gene expression is locally regulated in the axon in the context of regrowth.

The immediate follow-ups of this project will be (i) to uncover the molecular mechanisms of ribosome axonal supply; and (ii) to test whether increasing local ribosome supply in the axon improves its regrowth capacity. The present project will help determine whether a ribosome-specific transport exists, thus having a major impact in the field of cell biology. It will also help determine whether manipulating ribosome axonal supply can unlock axon regrowth in the injured CNS and/or improve current regeneration strategies. So in the long term, our results will help identify new molecular targets and overcome the limitations of current models, by acting locally instead of inducing global cell changes. Ultimately, our results will set the building blocks to trigger efficient and reproducible axon regrowth, and tackle the next steps of CNS functional reconnection, including guidance, synapse formation and maturation.

Altogether, results of this project will bring opportunities to design innovative therapeutic strategies of CNS repair based on a new perspective of local regulation of gene expression in the axon itself.

2.3. Dissemination, exploitation and communication activities planned

Dissemination will be done by publishing results in an international peer-reviewed journal with open access. Codes will be deposited on appropriate repository websites (Github). Results will be presented at national and international conferences to reach experts in the fields of intracellular transport and biological imaging: the annual congress of the Exo-Endo Club, the International Symposium on Biomedical Imaging and Shaping Life, an international conference on developmental biology. We will also encourage the PhD candidate to participate in public outreach events such as the festivals Pint of Science and Brain's Awareness Week. These events allow us to raise awareness in the general public, notably in the youngest, about the importance of publicly-funded research in daily life and its impact on key biological, biomedical and health issues of the 21st century.

Finally, in the long-term, this project may set the basis for the design of new therapeutic strategies of CNS repair, for which we will seek collaborative opportunities with biotechnology companies and involve the CNRS patenting office and SATT Sud-Est.

3. IMPLEMENTATION

Work plan

Aim 1 Optimization of high-resolution imaging of ribosomes in regrowing axons

We have validated two probes to detect endogenous ribosomes. We will test four additional probes to identify the best candidates in affinity and brightness. As in our preliminary work using confocal microscopy (***Figure 3***), we will screen different dyes, such as bright photostable ATTO dyes. Optimization will be performed in post-natal day 0 (P0) explants, where axons contain high amounts of ribosomes *(8)*. Ribosomes will be imaged in axons in microfluidic chambers using TIRF for single-molecule detection.

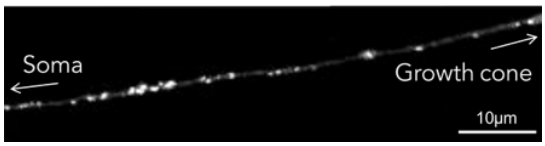


Figure 3 Confocal picture of a ribosome-targeting probe for detection of endogenous ribosomes in axons of a post-natal retina explant.

Aim 2 Development of an analysis pipeline for automated detection and trajectory tracking

Live imaging data will be analyzed with computer vision-based tracking. Results will be compared with a classical automated kymograph analysis *(19)*. An end-to-end pipeline capable of jointly detecting and tracking in complex biological image sequences will need to be developed. This framework should exploit temporal coherence by leveraging recent architectures with memory capabilities or attention-based models (*26*). Then, the incorporation of prior knowledge, and potentially biologically plausible constraints into the learning process is expected to improve model robustness. Similar strategies have already been explored in other contexts, for instance through the integration of physical constraints (*27*).

A major bottleneck remains the availability of annotated data. Such data are required to quantitatively validate the models, as this validation relies on the existence of ground-truth annotations — an extremely time-consuming process. To address this issue, we plan to investigate generative models, which could enable the creation of synthetic yet realistic video sequences, in a manner similar to approaches used in deepfake generation.

Finally, we will use Hidden Markov Modeling (HMM)-Bayesian statistical analysis to quantify movement parameters, including speed, directionality and transitions between active transport and passive diffusion (*28*). This approach is state-of-the-art for single-particle tracking (*28*) and well-adapted because it does not require a high number of events (few dozens) nor a long acquisition time (few minutes). Besides, this approach is robust, knowing the limitations of biological objects imaging (bleaching, drifting).

Aim 3 Correlative measurements of ribosome movements in relation to other organelles in axons

How ribosomes are addressed, transported and stored in the axon is currently unknown. To address this, we propose as a starting point to analyze ribosome transport in relation to other organelles. This includes membrane-bound organelles shown to regulate ribosome translational activity *(2)*, such as mitochondria (*29*), endoplasmic reticulum (*30*), lysosomes (*31*) and endosomes (*29*, *32*); as well as membrane-less organelles such as RNA granules (*6*, *33*).

To this end, we will perform multiplex imaging and correlative measurements of ribosome movements in relation to other organelles, using simultaneous acquisition with a dual camera system. The study of the dynamic organization between ribosomes and organelles, as well as their interaction, will provide an opportunity to develop so-called co-tracking approaches, that were previously used in human-object interaction (*34*). The correlation of the different transport parameters will be analyzed using the approach outlined in Aim 2. Finally, this aim will also be the opportunity to examine how the two ribosomal subunits move relative to each other, by labeling the two ribosome subunits with a different dye.

Aim 4 Data-driven modelling of axon regrowth capacity based on ribosome axonal supply

As a complementary theoretical approach, we aim to develop a mathematical model to predict axon regrowth capacity based on ribosome intracellular supply. Simulations of axon regrowth will be performed by integrating measured parameters of ribosome local supply (density, speed, directionality, pausing) in a model of advection-diffusion for axonal transport. Results will be confronted to experimental data using the different regrowth conditions in explant cultures (***Figure 4***). This fourth aim is exploratory and will be done in collaboration with mathematicians of the CENTURI community.

Expected results and risk assessment

This PhD project will have three key outcomes: (i) a widely usable tool to image endogenous ribosomes live; (ii) the development of a tracking method for axonal transport; (iii) the quantitative description of ribosome supply in the axon. Our data will set the basis of a mathematical model describing dynamics of ribosome local supply, providing a highly valuable insight into axonal ribosome transport, which is currently poorly understood. This model will be further used to identify key factors controlling ribosome axonal transport, e.g. RBPs, kinesins and dyneins, or the ER. This approach could be extended to study ribosome dynamics and storage in specific axon sub-compartments like in the growth cone in response to external cues.

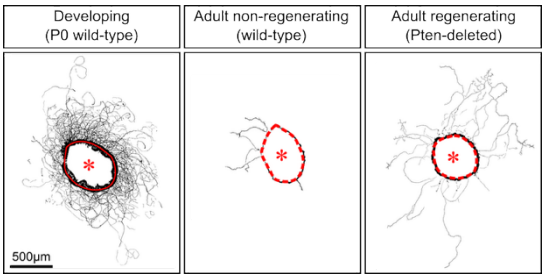


Figure 4 Beta-III-tubulin labeling of RGC axons with different regrowth capacities from retina explant cultures (data from Schaeffer et al., 2020). Retina explants are delineated by a red line and indicated by a red asterisk.

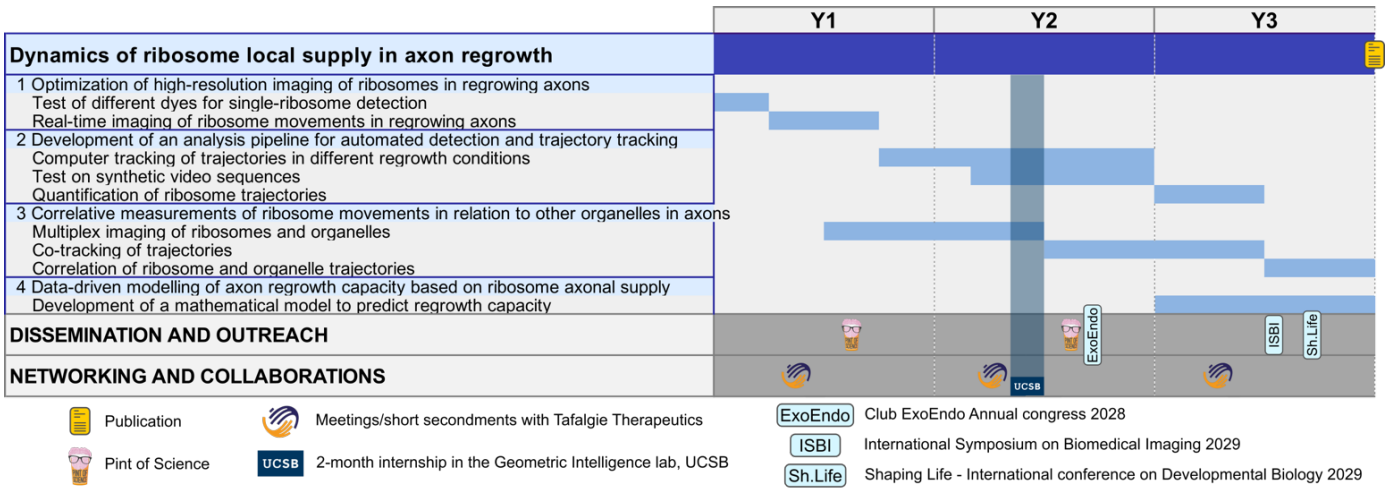
Retina explant cultures are routinely used in the lab, making the model set-up low-risk (*13*, *21*, *22*). Julia Schaeffer has extensive experience in optimizing intra-axonal live imaging (*13*, *35*) and in using micro-fluidic chambers (*36*), reducing data collection risk. Séverine Dubuisson has in-depth expertise in computer-vision image analysis and Bayesian modeling for particle detection and tracking (*23–25*, *37*, *38*).

A potential challenge is that ribosomes may be hard to detect in adult WT axons due to their low numbers. In this case, we will focus on ribosome density in fixed explants as a proof-of-concept and prioritize live imaging in adult Pten-deleted and P0 WT conditions. Imaging multiple objects may require optimization for resolution, interaction definition and correlation analysis. To manage this, we will also perform co-staining on fixed tissues, providing a starting point for studying ribosome transport mechanisms.

Feasibility and research environment

This project is scheduled on 3 years (see ***Gantt chart*** below). A post-doc specialist in super-resolution imaging has recently joined the Schaeffer team, and will be working in close collaboration with the PhD student for explant cultures and data acquisition. The IBDM has all essential facilities to carry out experiments, including an imaging facility with modules of live imaging (spinning disk, TIRF). The facility is run by 4 engineers and one scientific manager, our collaborator Pierre Mangeol, who are highly qualified with imaging questions and will help when encountering technical difficulties. Mouse lines (Pten-floxed), viral vectors and imaging reagents are available in the lab and surgical procedures have been ethically approved by the French Ministry of Research. Microfluidic chambers are generated in collaboration with Maxime Cazorla (INT), an expert of this technology.

Julia Schaeffer has been fully involved in training, supervision and successful PhD completion of 4 students – see CV (*2*, *13*, *21*, *22*, *40*, *41*). Séverine Dubuisson has successfully trained 8 PhD students – see CV (*42–46*). This ensures high-quality supervision of the PhD candidate and success of this research project.



4. ETHICS SELF-ASSESSMENT

Animals

Rationale and impact

This project addresses how local protein synthesis operates in regrowing axons of injured adult central nervous system (CNS) neurons, by focusing on the local supply of ribosomes, the functional units of protein synthesis. The hypothesis is that the regrowth capacity of adult CNS axons depends on local supply of ribosomes in the axon, and that this process is defective after injury, partly explaining the regeneration failure.

To test this, we will study the adult mouse visual system, a gold-standard experimental model of CNS injury and regeneration. The visual system is part of the CNS and recapitulates all molecular and cellular events of the brain and spinal cord, but is more accessible experimentally. Mouse visual system experimental procedures are well-established and relatively non-invasive, notably the intravitreal injection. This procedure will be used to inject adeno-associated virus type 2, which have a high tropism for retinal ganglion cells (RGCs). The project is based on ex vivo cultures of post-natal or adult retina explants. I have developed, optimized and extensively characterized this system in my previous work (*13*). It has the advantages to (i) recapitulate all features observed in vivo, and (ii) have high accessibility for downstream applications, e.g. real-time imaging in the axonal compartment.

Results of this project will have a major impact in our understanding of mechanisms underlying axon regrowth and regeneration. In the long term, they will benefit patients affected by chronic visual impairment and by traumatic or neurodegenerative disorders of the brain and spinal cord.

Animal justification

To test the project hypothesis, in vitro cultures of cell lines are inappropriate to recapitulate the CNS cellular and molecular complexity. Invertebrate organisms (Drosophila, C. Elegans) or anamniotes (fish) are not relevant either, as these organisms display spontaneous adult CNS regeneration, unlike mammals. Thus, rodent models are essential to explore mechanisms of axon regeneration and develop therapeutic strategies for CNS repair in humans.

Mice are appropriate to use in this project because the mouse visual system is similar to that of humans and allows to study all aspects of axon regrowth and reconnection (at molecular, cellular and circuit levels). As a mammalian organism, mice share many features with humans, notably adult CNS regeneration failure. In the axon regeneration field, the benefit of using mice is to go beyond the in vitro understanding of molecular systems and to have a global view in the framework of a whole organism. This is crucial if we want results to be eventually translated into a therapeutic approach for adult CNS injury in humans. Mice are very convenient thanks to transgenic methods developed in this species. This project will use Pten-floxed mice, which have been used in previous publications and do not present a known phenotype that requires special veterinary-related consideration. The optic nerve crush injury model has been well-established and studied in mice.

Summary of experimental procedures

Intravitreal injection for retina explant culture: 4-week-old mice mice will be anesthetized with an intraperitoneal injection of Ketamine (60-100 mg/kg) and Xylazine (5-10 mg/kg) and will receive an intravitreal AAV2 injection. A drop of tetracaïne will be applied prior to injection.

Post-surgery monitoring: Eyes will be covered with an anti-inflammatory medication and with an ointment (Ocry-Gel) during recovery from surgery. Mice will be placed on soft surface in normal position in warm environment for recovery and monitored until they are fully awake and their ability to walk is assessed. Mice will be monitored for any signs of suffering or pain right after surgery and once per day in the 48h following surgery.

Endpoint and euthanasia: 2 weeks post-injection, mice will be euthanized by CO_2 asphyxiation. Retinas will be immediately dissected and prepared for explant cultures. For post-natal retina explants, neonatal mice will be sacrificed by decapitation and retinas immediately dissected. Mice will be euthanized by CO_2 asphyxiation if they display any sign of illness or pain during the post-surgery period (e.g. weigh loss more than 20%, lethargy, behavioral abnormalities, eye infection).

Ethical considerations

All procedures of the project will abide by the 3R rule (Replace, Reduce, Refine).

The study of complex molecular and cellular mechanisms of regeneration requires the use of animal work and does not allow us to use replacement strategies like cell lines or organoids. Nonetheless, the project is largely based ex vivo procedures, as local translation in axon regrowth will be studied in post-natal and adult retina explant cultures. Thus, the research project replaces in vivo animal work with an ex vivo approach to focus on the axonal compartment. The development of a mathematical model will help predict axon regrowth capacity as a function of ribosome motility and distribution.

The number of mice will be greatly reduced thanks to this ex vivo system, which will allow to explore all molecular features of local translation. Both eyes of each mouse can be used, to further reduce the number of animals. The ex vivo procedures enable to prepare a high number of cultures (up to 30 explants from one mouse retina). The application of appropriate statistical tests will also help us to reduce the number of animals tested, as we will determine the minimal number of mice required to obtain satisfying statistical power before the experiment, and/or based on preliminary experimental data. Finally, we have a long-term experience in intravitreal injections and explant cultures, thus reducing the procedure-related losses and the overall number of mice.

Finally, refinement will be applied in all experimental mouse work all along this project by putting all attention to animal care (breeding, surgeries). We never noticed any pain or incomfort after the intravitreal injection (lasting less than 1 min per eye). This procedure is well-established and fully-mastered in the team. For surgical procedures and also for animal housing and manipulation, maximal efforts will be made to cause the least pain, suffering, distress or lasting harm to animals. Particular care will be brought to appropriate anesthesia, medication and monitoring during and after surgical procedures.

All animal work will be performed in close contact with the Ethics Committee of IBDM and with vets available for any support and advice (Dr Gaelle Odelin).

Legal authorizations

This project has obtained official approval by the Ethics Committee of IBDM and by the French Ministry of Research (authorized and registered under project number APAFIS #52331-2025020416358998 v3 (see below), valid until 2030. The use of genetically modified organisms (GMO) has been approved in IBDM in the mouse facility (A1-1949) and in the L1 cell culture facility (L1-1857). The rodent animal facility at IBDM has the approval number G1305521**,** starting in February 2022 and valid for 6 years.

As an experienced researcher, I have the License to perform animal experimentation on rodents (experimental design, project license writing, mouse colony management) (obtained June 2018 and updated with specific training every year) and the License to perform surgical procedures on rodents (obtained November 2024). The PhD candidate will be provided training to obtain the License to perform animal experimentation on rodents for post-natal explant cultures, and will work in collaboration with a post-doctoral researcher who has the License to perform surgical procedures for the surgeries for adult Pten-deteled explant cultures.

Artificial Intelligence

Rationale and impact

One objective of the project is to develop an automated analysis pipeline for detecting and tracking biological objects in live imaging data using computer vision, deep learning, and generative models. The pipeline will integrate advanced architectures (e.g., attention-based models) and prior biological knowledge to improve robustness. To address the lack of annotated data, synthetic video sequences will be generated. This method will have a key added value in the exploration of our biological question and will significantly advance both fields of axon biology (axonal transport) and computer vision (single-particle tracking, multi-objects tracking).

Ethical considerations

In this project, the use of biological imaging data does not include sensitive or proprietary information. Synthetic data generation or model training may present a risk of bias, potentially leading to skewed or

unrepresentative results. To address this, diverse and biologically plausible synthetic datasets will be created, validated, and compared against real data to ensure representativeness and fairness. Synthetic data will be used solely for scientific validation and clearly labeled as such. Any public release will include disclaimers and metadata to prevent misuse.

Complexity of deep learning models may hinder transparency, making it difficult to interpret or reproduce results. Models will be documented thoroughly, including architecture, training data, and parameters. Code and datasets (where possible) will be shared openly or with collaborators under FAIR principles.

Training deep learning models can consume significant computational resources, contributing to carbon emissions. Energy-efficient training protocols will be adopted, such as using cloud providers with carbon-neutral commitments and optimizing model architectures for computational efficiency.

Incorporation of biological constraints and prior knowledge must be scientifically justified to avoid misleading conclusions. Biological constraints will be validated by using observations and literature review, and models will be cross-validated with experimental data.

The development and use of AI models is of minimal risk regarding the EU AI Act, and will nonetheless adhere to the Act regarding transparency, risk assessment, and dual-use safeguards.

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