Universidad Carlos III de Madrid

Bachelor's Degree in Biomedical Engineering

Bachelor Thesis

Study of the expression of purinergic receptors by SPIM microscopy in a transgenic model

Author

Cristina Labajo Villaverde

Tutor

Manuel Desco Menéndez

June 2017

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1. Introduction

1.1 Motivation

The brain is the most important and complex organ in the body, so much so that, it is not known how it works exactly yet, making it appealing for researchers all over the world. The researchers are focusing their efforts in finding out how it works, developing new tools and techniques to reveal how the neurons interact and how the connection network between these neurons is distributed among the brain.

Using several advances in science, such as transgenic animals or some new tools like the Single Plane Illumination Microscopy, new models can be made that enlighten these questions. Also taking advantage of the possibilities offered by the SPIM, being the 3D reconstruction of large portion of tissue its main utility, and with the help of techniques as the immunohistochemistry, it would ease a major understanding of the brain that could help in a better handling of vascular diseases.

1.1.1 Description of the project

The project is framed under a multidisciplinary collaboration with several institutions. The global objective of the project is to develop new tools that improve the field of the computational systems inspired by the organization of the brain.

In particular, new methodologies for the data acquisition are being used to define analytic models and simulations of the cerebral architecture from an anatomical and functional point of view in two different organizational levels, cerebral regions and neurons.

With that purpose in mind, the team that is carrying out this project is studying the neural connections that takes place in the brain by focusing in the purinergic receptor P2X7 [1], an ATP receptor, as an indirect measure of electrical activity. The study is made in a mouse model where the P2X7 receptor is labelled with a Green Fluorescent Protein (GFP) via genetic engineering. The Laboratory of Medical Image is in charge of studying the location of these receptors inside the brain and its evolution during the development.

The P2X receptors are a huge family of nucleotide receptors that are widely distributed among the brain. It has been also discovered that they participate in a very important manner in the synaptic event, in particular in the neurotransmission mediated by the

nucleotide ATP (Adenosine triphosphate). P2X receptors interact with the ATP molecule and as a result of this interaction they are a key control in the secretion of neurotransmitters and signal transmission.

In the transgenic model that express fluorescence, studies have been made using fluorescence microscopy, more specifically Confocal Microscopy and Single Plane Illumination Microscopy, in order to locate these purinergic receptors, observe its distribution in the brain and obtain neuronal activity patterns.

1.2 Fluorescence principle

Fluorescence was first described by the British scientist Sir George G. Stokes in 1852 [2]. It is a phenomenon that takes place at a molecular level and consists of emitting light almost simultaneously to be reached by a beam of light coming from another source and during the time the exposure to that source lasts. The processes of absorbing the light and sending out radiation are called excitation and emission.

There were more investigations during the XIX century which proved that many specimens emitted fluorescence when ultraviolet light felt on them but until the 1930s this technique was not used in the biological environment.

Fluorescence consists on exciting a sample with light, the sample absorbs and then releases the light [3]. The energy of this radiation light collides with the atoms when the photons interact with the molecules. The photons may be absorbed, exciting the electrons to a higher energy state. When the electron goes back to its natural energy state it emits a photon and since the emitted photon has a lower energy than the absorbed it means it has a longer wavelength. This fact had been noticed by Stokes when he discovered the fluorescence, the emission always took place at a higher frequency (longer wavelength) than the excitation.

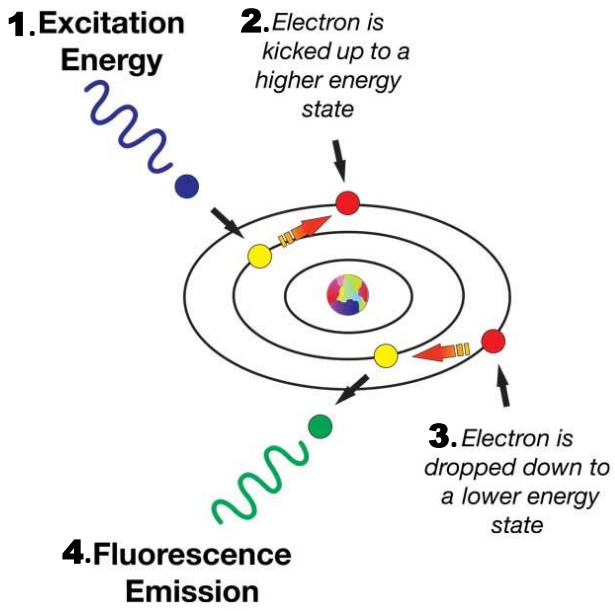

Figure 1.Principle of fluorescence [4].

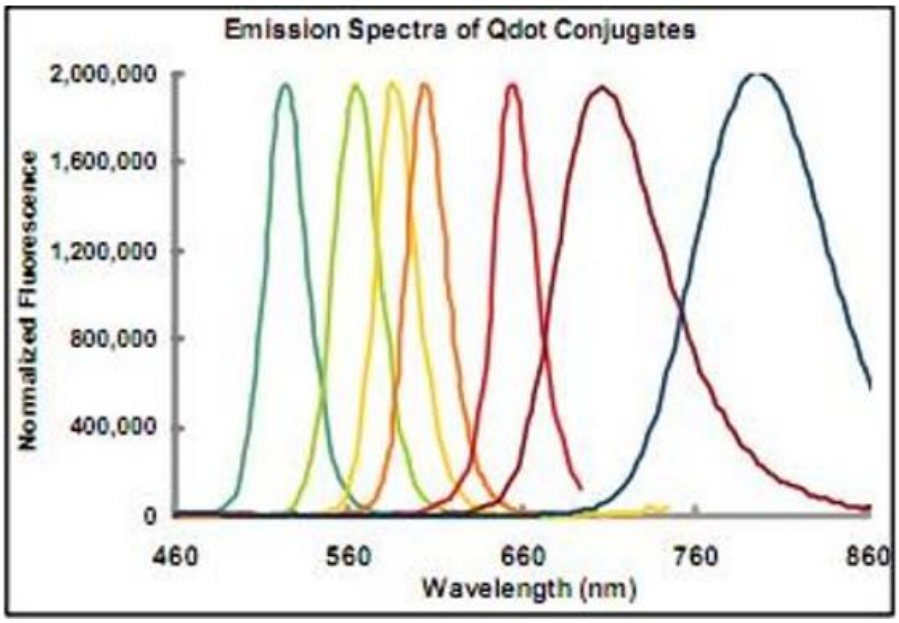

Figure 2. Wavelengths and lasers (courtesy of Jorge Ripoll).

But not all the samples emit fluorescence. The fluorescence can come from different sources. It can be the product of bioengineering when the genetics of the animals are modified to express fluorophores or after the extraction of the tissue, when some molecules can be labeled with fluorophores in a process known as immunohistochemistry. It also exists some species that shows fluorescence in a natural form.

1.2.1 Transgenic organisms

Using genetic engineering, which consist in modifying the genome of an organisms with the help of modern technologies, is possible to label some proteins in order to express fluorescence [5]. The most common fluorescent protein used for transgenic organisms is the GFP.

The Green Fluorescent Protein was discovered by the professors Martin Chalfie, Roger Y. Tsien and Osamu Shimomura. It is a protein produced by a jellyfish, the *Aequorea Victoria.* This protein emits at the frequencies that belong to green color. The gene that codifies this protein can be isolated and since its discovery is widely used in molecular biology as a marker.

The gen that codifies for GFP is introduce in the genome of other species ligated to other proteins of interest. This method is used to visualize processes that were invisible before, such as the development of neurons, how tumor cells behave or the proliferation of the HIV.

The advantage of this method is that it provides the specimen with an intrinsic fluorescence, the fluorescence is incorporated in the DNA of the animal and can be produced spontaneously in the cells that include the gene where it is codified without needing other agents. It can have other applications as in the case of the tracking of a protein during the development of an embryo, by introducing the GFP in the early stages and being able to observe the formation of different structures.

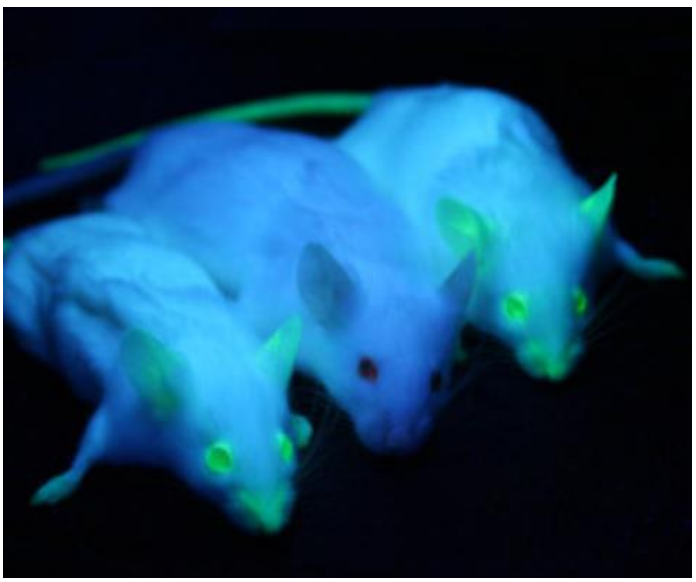

Figure 3. Transgenic mice [6].

1.2.2 Immunohistochemistry

Via multiple fluorescence labelling several molecules can be identified simultaneously by different probes giving a map of its distribution among the tissue. The process that leads to the multiple labelling is called immunohistochemistry. Immunohistochemistry (IHC) is a technique that is composed by anatomical, biochemical and immunological procedures. It consists on the labelling of specific antibodies that interact and bind with the antigens that are interesting for a study in particular. There are different types of antigens, they can be proteins or parts of proteins that are expressed only in a certain type of cells, DNA, RNA, etc. and they can be located everywhere inside the cell or in the cellular membrane, although when they are inside the nucleus a previous treatment is required in order to increase the permeability for the antibodies to reach their destination. Using this technique certain molecules within cells can be located and the distribution can be mapped, and it is also possible to see the organization of a tissue.

A primary antibody is bound to a specific antigen that binds a certain cell type. In order to be able to visualize this target cell a secondary antibody labelled with fluorophores is added to attach the primary antibody [7]. This secondary antibody recognizes the animal species which the primary antibody comes from. For example, if we want to mark the protein GFP we would use a primary antibody anti-GFP made from mouse. If we want to see this primary antibody we would have to add a secondary antibody that recognizes the previous species, it would have to be from another different species and have fluorophores with a certain wavelength, for instance anti-mouse 488 made from donkey. If we are labelling more than one antigen at the same time we need to be very careful not to repeat and mix the species and the wavelengths of the fluorophores. In the following figure we can see graphically how this process would work.

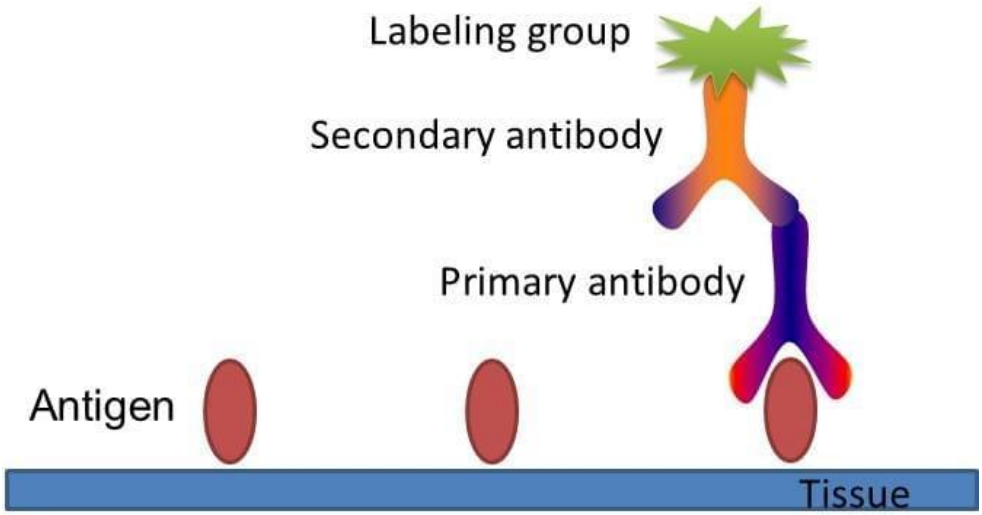

Figure 4. Principle of immunohistochemistry [8].

1.3 Fluorescence Microscopy

The microscopy has been an essential instrument in the scientific field, since its creation by a Dutch spectacles manufacturer, Zaccharias Janssen around 1590, until this days where we can find a huge number of different microscopes. Among all the different models we have used the fluorescence microscopy.

In the 1930s, it became a very important tool for scientists which allowed them to stain tissue components with a high specificity, enabling them to visualize structures that were invisible before under the traditional optic microscopy, locating them in the inside of the cell and studying possible molecular interactions.

There are different kind of microscopes that use the principles of fluorescence to take images, being the epi-fluorescence microscope the most widely extended. They use incident light from above and the objective is used as the illumination condenser and the collector of fluorescence [9]. The essential components that these microscopes need to have are:

- A light source
- A filter that allows only the wavelengths of interest to pass through it.
- A dichroic beam splitter or mirror to separate light by transmitting and reflecting light as a function of wavelength.
- A filter that only let the light radiation coming from the sample to pass.
- A charge-coupled device (CCD) camera that captures the fluorescence.

With the epi-fluorescence microscope it is possible to detect single molecules that emit fluorescence among the tissue that do not emit. It has some advantages but also some drawbacks: The sample needs to be sectioned in very thin slices, the light beam has a low penetration on the tissue and the camera captures all the fluorescence coming from the entire sample, not only the plane that is focused, which produces a lot of noise.

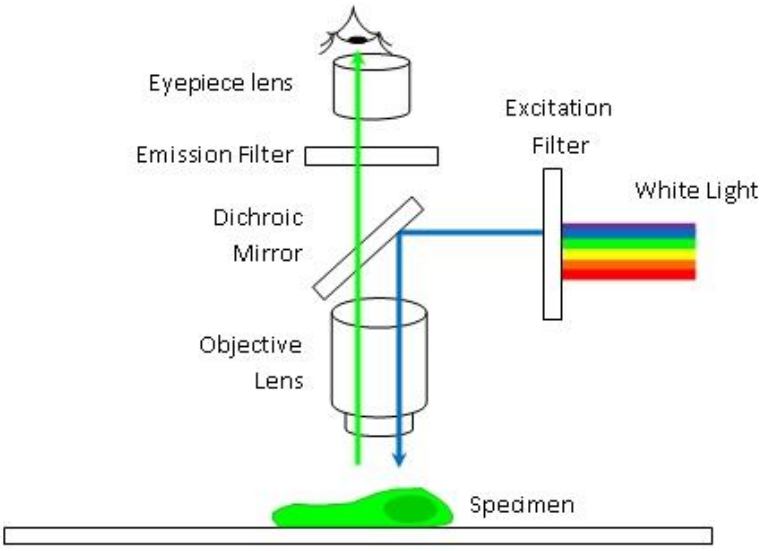

Figure 5. Epi-fluorescence microscope configuration [7].

1.3.1 Selective Planar Illumination Microscopy

Selective Planar Illumination Microscopy (SPIM) is one of the newest forms of microscopy at this moment. It has supposed a revolution in the area of research because it allows investigators to obtain images of whole organs with high resolution instead of having to laminate them physically, which often produce image artifacts and changes in the tissue. The SPIM has been designed with certain characteristics that allows it to achieve these results. It is based on the fluorescence microscopy, illuminating a sample with a laser, but instead of a beam, the form of the laser that reaches the sample is a planar sheet [10]. This effect is achieved due to the presence of a lens with a cylindrical form that causes the beam to be reformed, as it passes through the lens, into a planar sheet that illuminates the sample from the side only in a unique plane, which is the one that emits the fluorescence recorded by the detection objective. This technique has many advantages such as reduced photo-bleaching, because unlike other forms of microscopy such as the confocal microscope the sample is selectively illuminated, only the plane of interest is reached by the excitation laser, leaving the rest of the sample unaffected, and allowing to make more studies and radiating the sample more time.

Another advantage is the fast acquisition that makes possible to do a more extensive and complete study by repeating the image acquisition from different angles.

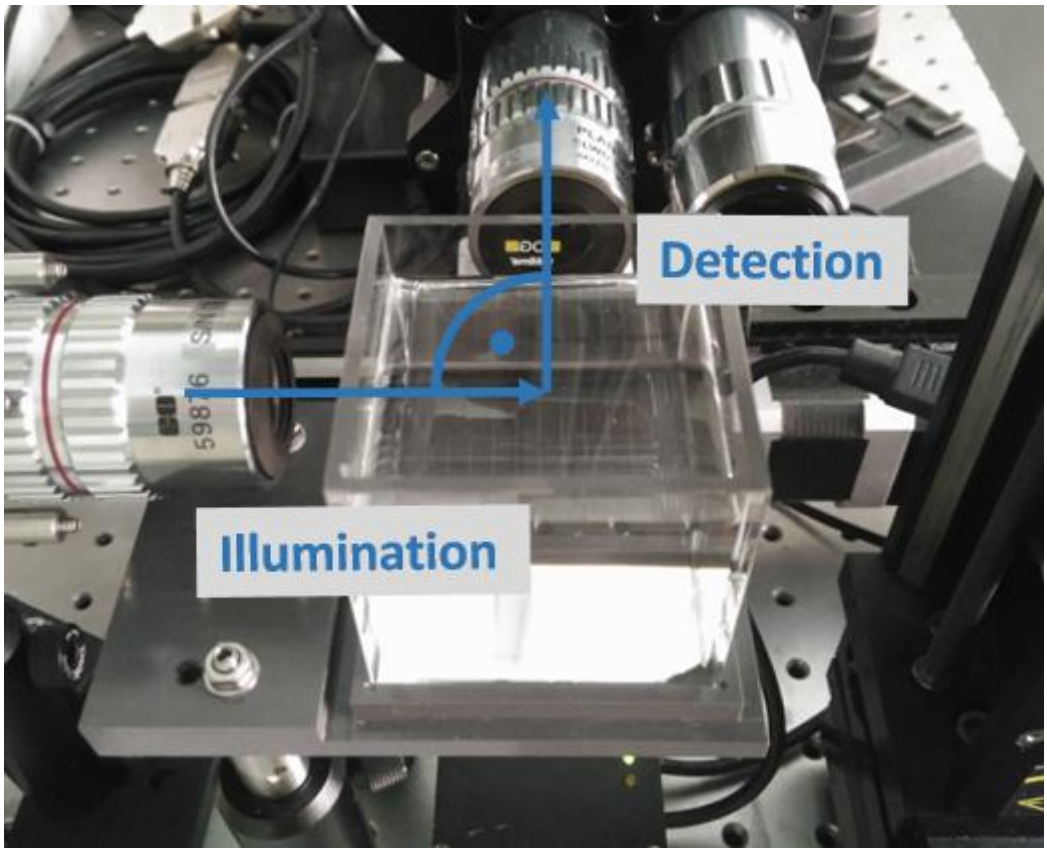

Figure 6. SPIM's objectives.

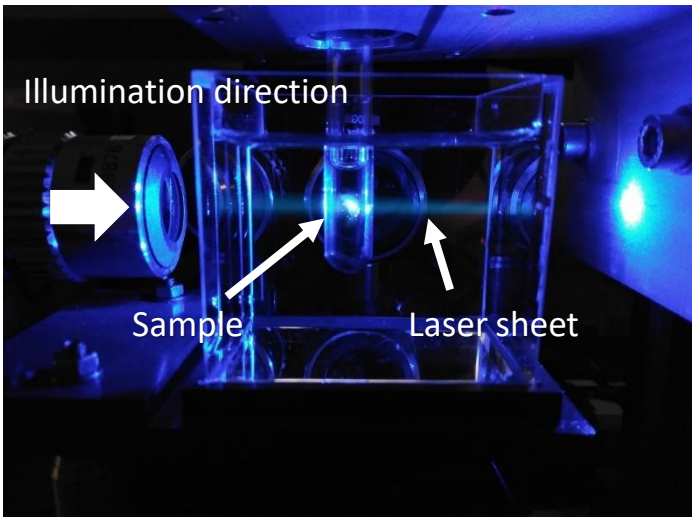

Figure 7. SPIM microscope emitting the light sheet.

In the SPIM the directions of excitation and acquisition are place orthogonally and the portion of tissue that is illuminated in a plane is placed to coincide with the focal plane

of the objectives that detect the fluorescence emitted, which is the intersection of the illumination and detection axes.

Whereas the excitation planar sheet is immobile, the sample is moved in the z direction to produce a three-dimensional stack of parallel images taken with a certain distance between them, the minimum distance is determined by the thickness of the light sheet itself and by the precision of the motor that is in charge of displacing the sample along the detection axis which has a high precision. The thickness of the laser sheet and the precision of the z direction motor are the features that determines the axial resolution of the image. Another motor is in charge of rotating the sample allowing to obtain different views. Also, the width of the light sheet is the one that defines the portion of tissue that is illuminated and therefore captured by the camera.

Ideally the light sheet would be uniform but this is not the case in the reality where it is shaped in a Gaussian-like form. To minimize this effect the sample is placed in the location where the light sheet is narrower and more uniform. Several lasers are commonly used in light-sheet microscopy to excite the fluorophores in the sample, being the ones with wavelengths 560 nm or 488 nm the most common.

The SPIM has several lasers, each of them with different wavelengths that are controlled with an acousto-optic tunable filter (AOTF) which is often used to precisely control the wavelength, power and timing of the illumination. Then they are directed through dichroic mirrors that selectively reflects one or more parts of the light spectrum while transmitting the rest of the spectrum and when the sample is excited and emits fluorophores, the radiation goes across an emission filter that only let the light of a certain wavelength pass. These filters are coupled in a filter wheel that is controlled with a software and that changes depending on the exciting laser used.

After the samples are excited with the laser sheet, a charge-coupled device (CCD) camera collects all the fluorescence from the sample at once without the need for raster imaging [11]. This design makes data acquisition in light-sheet microscopy extremely fast and efficient in comparison with confocal microscopy and other scanning microscopes.

This microscope allows taking images with cellular resolution. The spatial resolution is defined as the minimum distance where you can distinguish two consecutive points on the same specimen as separated entities. The numerical aperture also influences in the resolution. The numerical aperture (NA) is a measure of the ability to collect light and resolve sample detail at a certain distance. The higher the total numerical aperture, the better the resolution.

Another feature that can control the resolution is the wavelength of the light beam used to image the sample, shorter wavelengths are able to perceive details more accurately than longer wavelengths.

The resulting formula that explains the image resolution in terms of NA of the objective and wavelengths (λ) of the laser that excites the sample is the Rayleigh equation:

$$\text{Resolution } (r) = 0.61\lambda/NA$$

The SPIM is a very useful too because the several lasers, each of them emitting in a different wavelength, can be used in the same portion of tissue and with the immunohistochemistry techniques it is possible to select different targets which would result in a very complete image making it able to offer multiple possibilities.

1.3.2 Confocal microscopy

Confocal microscopy was patented by Marvin Minsky in 1957 and it is a form of fluorescence microscopy similar to the epi fluorescence microscope but with some peculiarities that improve the images. It has high resolution that is provided thanks to a spatial pinhole added to eliminate out-of-focus fluorescence.

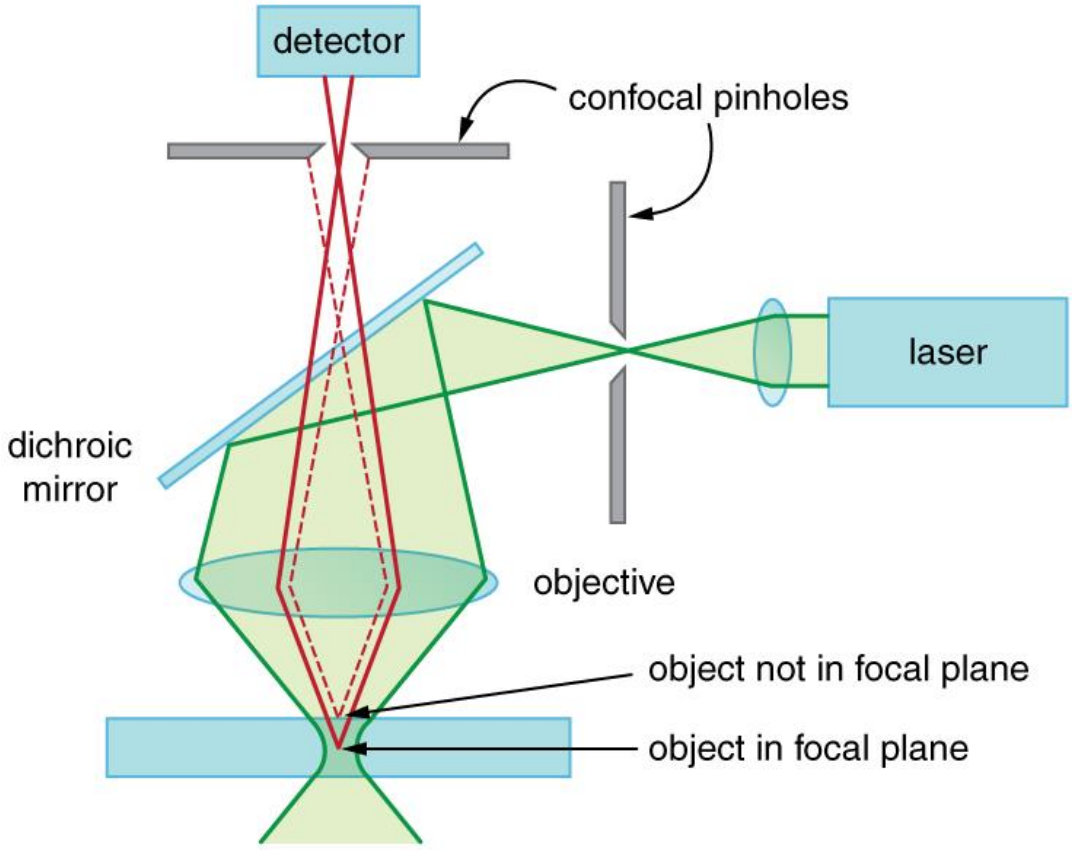

Figure 8. Confocal microscope configuration [12].

The pinhole is a hole located in front of the photomultiplier that stops the radiation of the regions that are not on focus acting as a spatial filter, the radiation from the regions located above or below the focal plane do not converge on the pinhole and therefore they are not detected by the photomultiplier. With this configuration 2D images are obtained at different depths, scanning different focal planes along the z direction resulting in a stack of images.

First, the width of the laser beam is augmented to be focused afterwards by a lens, concentrating it on a small point at the focal plane, which is different from the epi-fluorescence microscope that concentrates the wide beam on a large volume of the sample, causing that a lot of radiation is received by the photomultiplier and it detects a lot of noise, reducing resolution and image contrast.

Confocal uses point illumination, avoiding to illuminate the complete sample every time it takes an image, but this decrease the intensity which is translated in longer times of acquisition.

Even though the quality of the image is improved, the confocal microscope presents some drawbacks. As mentioned before the times are longer and, as the sample is scanned point by point forming a raster image the photo-bleaching also increases by the long exposure of the fluorophores to a high intensity laser.

As the SPIM, it allows three-dimensional reconstructions by taking images at different depths but the beam of light has a lower penetration than in the SPIM, visualizing narrower sections. The maximum penetration of the confocal microscope is around 100 µm [13].

1.4 Tissue clearing method

One of the main problems of fluorescence microscopy is that the laser sheet needs to penetrate the sample in order to excite the fluorophores located in the inside [14]. A phenomenon called scattering takes place, which consists in the deviation of the light sheet from its straight trajectory due to the presence of non-uniformities in the medium that the light sheet has to travel by. Some species do not have this problem, since there are some animals, for example, the zebra fish, that are transparent and the light is not scattered, therefore some in vivo experiments are taking place at this moment taking advantage of this peculiarity [15].

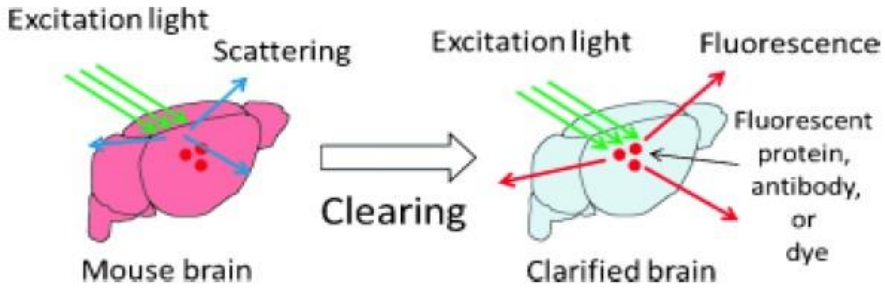

Figure 9. Principle of tissue clearing [16].

In order to avoid this effect two different resolutions can be found, either it is needed to make the samples transparent to allow the penetration of the light sheet through the tissue or to cut them thin enough. Our aim is to visualize whole mouse brains that is why the method that better fulfill our requirement is the clearing processes.

The objective of the clearing protocols is to make possible for the light sheet to penetrate through the sample and for the radiation emitted by the fluorophores, once they are excited, to come out and reach the detection objectives. In order to achieve this, the samples need to be transparent enough by using a mixture of chemical agents. The molecules that contribute most to have light scattering are the lipids, therefore the removal of the lipids is the main objective of the different clearing methods.

Another issue to take into account is the refraction of light. Light scattering occurs at the boundaries of the sample between the tissue and the immersion oil because they have different refraction index, which describes how light moves through a medium, therefore it is needed that in the cleating protocol the equalization of the refraction index inside the specimen takes place for the light sheet to penetrate straight without deviations.

There are several clearing methods that have been proven to work such as FOCUS CLEAR, SCALE, CLARITY, BABB or CUBIC, which test different mixes of organic solvents, detergents, alcohols, sugars, etc. The aim is to achieve a high degree of transparency but at the same time it is necessary to preserve the functional characteristics and the anatomy of the tissue unchanged. A swelling of the tissue has been proven to be common to all the clearing techniques, but as it does not affect the anatomical properties, it is not taken into account.

Two aspects of the different methods to be valued are the compatibility with the immunohistochemistry and the period of incubation with the chemical agents. The combination of chemical agents can be aggressive with the fluorophores, bleaching them and causing a loss of the radiation needed to take the image. Some of these

methods have been proven to respect the fluorophores, being compatible with the IHC, such as CLARITY or CUBIC.

Regarding the duration of the incubation some of these methods can be very time consuming, the whole clearing process may take up to several months as in the case of SCALE. Some of them also required difficult procedures that are very laborious to repeat.

1.4.1 CUBIC method

The method chosen to clear the mice brains was the Clear, Unobstructed Brain Imaging Cocktails (CUBIC) clearing method which is relatively fast, it takes around two weeks and it only involves two different reagents making it simple, apart from the fact that the components of the reagents are not expensive [17]. Following the CUBIC method whole organs become transparent without bleaching the fluorophores which allows to take the images with SPIM in a highly efficient way. The protocol consists on the incubation of the sample with a mixture of sucrose, urea, alcohols, detergents and water that clear away the lipids and homogenize the refractive index.

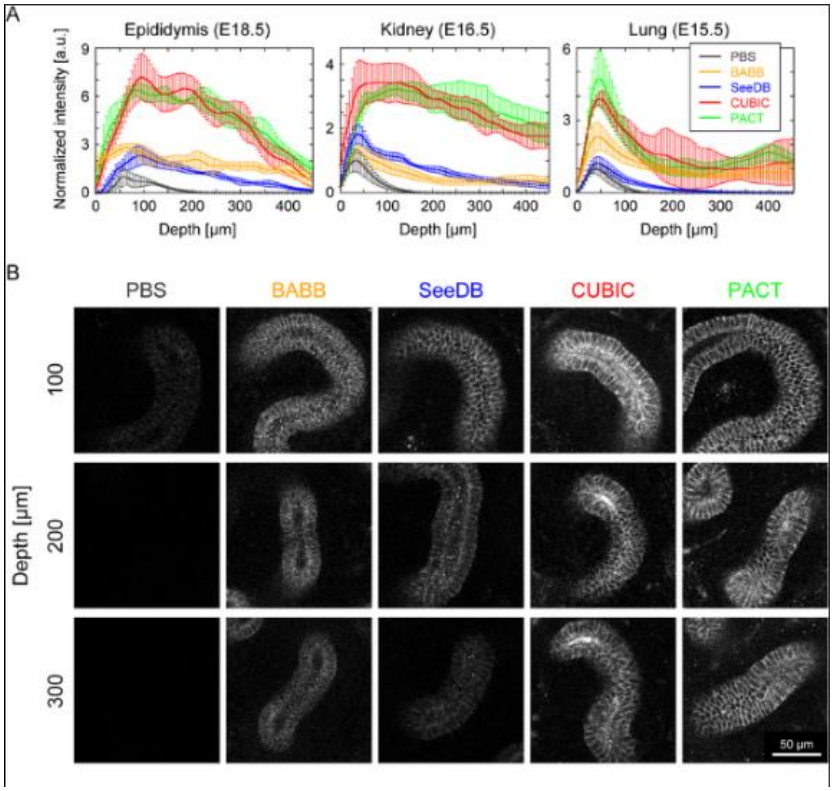

Figure 10. Comparison among different tissue clearing methods [18].

2. Materials and methods

2.1 Tissue perfusion and fixation

In order to study what happens in the brain we need to preserve it in a life-like state before it starts to degrade and decompose. But the fixation of whole organs has a difficulty because the normal fixative methods that work with smaller portions of tissue don't penetrate the entire organ at the same rate leaving some parts unfixed or fixing them once the cells are dead. In this method the circulatory system of the own animal is used to pump and distribute the fixative for all the body, reaching all places quickly [19]. It is necessary that the fixative enters the body of the animal with a pressure similar to the physiological one if we don't want to damage the circuit. This physiological pressure is different for each species. For this study we have chosen a perfusion procedure using 4% paraformaldehyde (PFA) injected transcardially through the left ventricle in order to fixate the brain in the most uniform and quickest way, which is the most popular procedure.

The mice are sacrificed in different stages of development to study the embryonic tissue and the maturation of neuron connections. First of all, the material for this procedure is prepared in a ventilated cubicle, because the gases produced in the preparation and manipulation of PFA are toxic. The material we are going to need to prepare the PFA solution and perfuse the mice consists on containers, absorbent underpads, filter papers, magnet, magnetic stirrer, thermometer, pH indicator, surgical instruments and a peristaltic pump.

Before we begin with the perfusion process the animals have to be properly anaesthetized and completely free of sensitivity. The anaesthesia used is a mix of xylazine and ketamine specifically calculated taking into account the weight of the animal. It is injected following the intraperitoneal route. In order to know if the rat is correctly under the effects of anesthesia, we check its foot reflex. Once we have the guarantee that the animal has been properly anaesthetized and it is not going to suffer we can continue with the perfusion.

Perfusion Protocol

The animal is fixed to a board making sure it is straight. Once we have it immobilized by the paws we proceed to make a cut in the skin of the abdomen. Once we have open the skin we cut into the muscle also, being careful of not pricking or tearing any

organ, if this were the case the vascular circuit would be disrupted and the perfusion would not be satisfactory. Once we have open the abdominal wall, we face the diaphragm that we have to tear without touching the lungs, we do this by using the curved, blunt scissors, starting on one of the laterals. Then we make a cut through the rib cage, lifting it and we hold the sternum upwards. We pierce the heart through the apex inserting the needle through which we are going to introduce the fixative and we make a small incision in the right auricle that is going to be the evacuation route for the blood in the circulatory system.

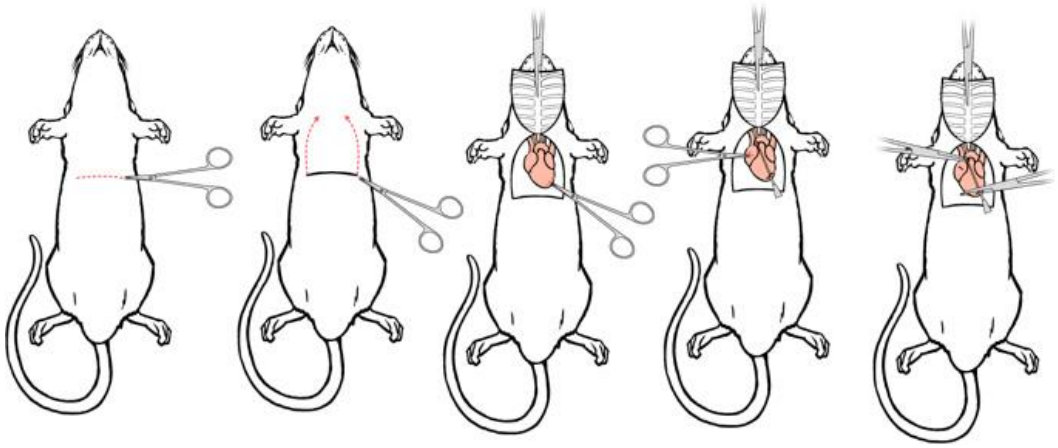

Figure 11. Diagram of perfusion protocol [19].

We set the peristaltic pump to 30 ml/min. Firstly they are perfused with PBS (pH=7.4) to dilate the capillaries and to wash the blood out. After that, they are perfused with the fixative, PFA 4%, the brain was carefully extracted and left immersed in fixative solution at 4°C overnight for a correct post- fixation, then the samples were transferred to PBS 1x solution.

Also, to optimize the method to visualize the vasculature of the brain and see the relation of the purinergic receptors with the vasculature, some wild type mice where perfused with Fluorescein isothiocyanate (FITC) – lectin from Lycopersicon esculentum (Tomato) that is used to label endothelial cells [20], being the perfusion protocol slightly different: Mice were sacrificed by CO2, then they were transcardially perfused with Phosphate buffer ad heparin for eliminating the blood of the vessels, then they were perfused with PFA 1% and after the PFA, 10 ml of FITC-lectin are introduced allowing the lectin to incubate for 2 minutes and finally PFA 4% is introduced.

2.2 Tissue sectioning

Even though the whole organ could be cleared with the CUBIC clearing method the bigger mice's brains where sectioned dividing the sample in three parts, the two cerebral hemispheres and the cerebellum. This division was made in order to allow the reagents of the CUBIC protocol to enter faster into the sample and make the brains transparent in less time and because it is easier to visualize the brain if it is divided in parts, not only because the accessories of the homemade SPIM that is available are limited but also because the SPIM artifact that has to do with illumination gradient changing as the distance that the light travels inside the sample increases. This sectioning is performed manually.

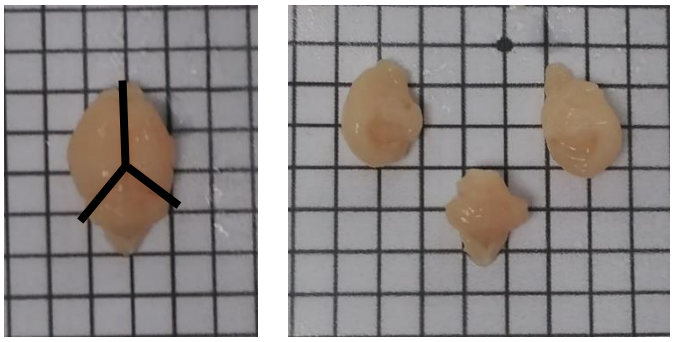

Figure 12. Sectioning performed in mice brains.

For other applications of the SPIM such as the study of vasculature, the brains were sectioned coronally in slices of 2.5-3 mm of thickness with the help of the vibratome (VibratingMicrotome 7000smz-2, Campden Instruments Ltd, Loughborough, UK) to ensure that the cuts were straight in order to avoid future artifacts, because when the surface of the sample presents a lot of irregularities lasers are prone to suffer scattering.

In order to place the tissue in the vibratome it has to be embedded in agarose 1.5% to prevent the organ from moving while the slicing process and also to maintain the proper orientation of the sample. In order to cut them always in the same way we place the brains with the cerebellum on the bottom causing the cuts to be coronal and sectioning the tissue from the rostral to the caudal side of the sample. Once the slices have been obtained we place them in PBS 1x and leave them at 4 °C.

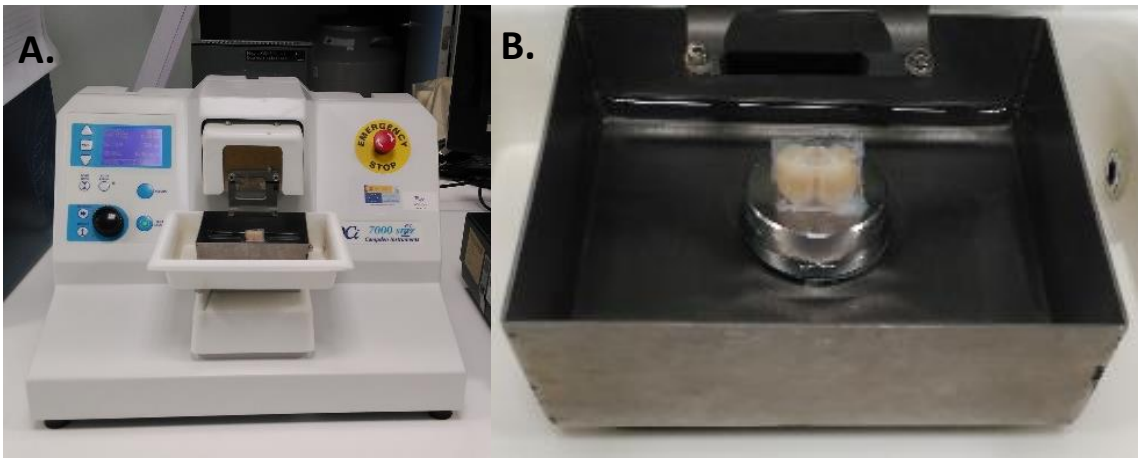

Figure 13.A: Vibratome used to slice the brains of the mice perfused with lectin. B: Brain inside an agarose block ready to be sliced.

2.3 Immunohistochemistry analysis

SPIM microscopy is based in fluorescence. There are different ways for a molecule to emit fluorescence. It can be born with the fluorophores, thanks to the genetic engineering where they modify the genes of the rodents to produce a certain protein that emits fluorescence, or an immunohistochemistry needs to be perform.

The immunohistochemistry consists of two periods where the sections are incubated with antibodies. On the study of the purinergic receptors the IHC is not necessary although sometimes it can be used to enhance the intensity of the intrinsic fluorescence. In these cases, α-GFP from chicken is used as primary antibody and incubated during 3 days. Then α-chicken 488 from donkey is used as secondary antibody conferring the fluorophores that will be used in the visualization.

The GFP was ligated to the purinergic receptor P2X7 related with the intake of ATP and known to play an important role in the communication between neurons.

In the study of the vasculature, apart from the lectin which was perfused and marks epithelial cells in the capillaries, it is interesting to label other parameters, for example, the young neurons that are tagged with the antibody doublecortin that has a cytoplasmic expression and also stem cells where marked with Sox2 which is identified in the nucleus of these cells.

Immunohistochemistry Protocol

After the samples have been incubated in the reagent 1 of the CUBIC method the primary antibodies are dissolved in a blocking solution, in a concentration 1:200. They are incubated in the incubation shaker at 37 °C for 3 days and nights and in agitation at a speed of 80 rpm. After that they are washed with PBT 0.1% on a shaker at room temperature, three times during 30 minutes each time, then they are incubated with the secondary antibodies dissolved in a blocking solution specific for the secondary antibodies and then would spend the same amount of time than with the primary antibodies, 3 days straight in the shaker at 37 °C.

Primary Antibody	Secondary antibody
Chicken α-GFP	Donkey α-Chicken 488
Goat α-Sox2	Donkey α-goat 594
Goat α-DC	Donkey α-goat 594

As the last two primary antibodies are from the same species they cannot be used together because the secondary antibody would bind both of them without doing any distinction and a false positive would be obtained. Sometimes we would mark the samples with sox2 and sometimes with DC.

2.4 Tissue clearing process using CUBIC protocol

In previous works in the Laboratory of Medical Image [21] [22] several clearing methods were tested to get to one method that would be fast, cheap and compatible with the immunohistochemistry. The following table is a comparison between the different methods (BABB [23], Scale [24], Clarity [25] and CUBIC [17]) with their respective advantages and disadvantages:

	ADVANTAGES	DISADVANTAGES
BABB	• Short duration (2 days) • Easy to reproduce • Economic	• Quenching of the fluorophores
FOCUS CLEAR	• Short duration • Easy to reproduce	• Very expensive
SCALE	• Easy to reproduce • Economic	• Long duration (months)
CLARITY	• Works well with the IHC	• Long duration (2 months) • Sophisticated, difficult to reproduce.
CUBIC	• Medium period of time (2 weeks) • Easy to reproduce • Economic • Works well with the IHC	-

The chosen method was the CUBIC, used to make the samples transparent. The protocol lasts 14 days for whole mouse brains and the reagents used are not expensive, in addition the CUBIC protocol is compatible with the IHC, in fact the immunohistochemistry is performed at the same time than the clearing process.

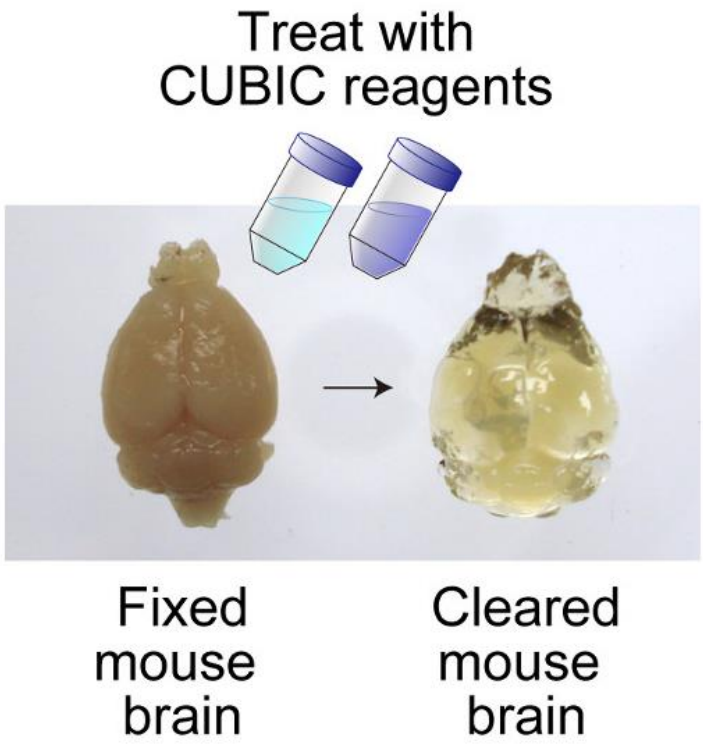

Figure 14. Mouse brain cleared using CUBIC method [26].

Several species have been cleared already in the Laboratory of Medical Imaging [27], which saved a lot of time when deciding which method to use or how long the protocol should be, even though the protocol for different stages of mice brain had to be optimized.

The CUBIC protocol consists of two different reagents each of them with a different function. The CUBIC reagent 1 (R1) is used to remove the lipids from the tissue which would make the tissue transparent. It is a mixture of the following chemical ingredients:

- N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine
- Distilled water
- Urea
- Triton X-100

The N, N, N', N'-tetrakis (2-hydroxypropyl) ethylenediamine with molecular formula C14H32N2O4 is a polyalcohol and it is in charge of removing the lipids that contribute to the opacity of the sample. It also decolorizes the possible remains of blood that could persists after the perfusion and solubilize the tissue. The Triton X-100 is a detergent used to permeabilize the cellular membrane allowing the diffusion of the polyalcohol inside the cells. To solubilize the proteins and help to maintain them intact the urea is needed paying attention to the concentration, it has to be enough to preserve the proteins and maintain them without changing their configuration.

The CUBIC reagent 2 (R2) is the responsible of equalizing the refractive index (RI) inside the sample and with the immersion oil where the probes are place, inside the special glass container to approximately 1.48-1.49. R2 consists on a mixture of the following substances:

- Sucrose
- Distilled water
- Urea
- 2,20,20-Nitrilotriethanol

CUBIC Protocol

The CUBIC protocol for mouse last 13 days approximately. It starts the incubation in R1 and for three days the sample is immersed on it at a temperature of 37°C, with agitation at 80 rpm. When the 3 days has past the R1 is changed by new one and DAPI is added at a concentration 1:2500. DAPI is a nuclear staining and since it is a

fluorophore that is bleached with light, from the moment it is added the samples must be conserved in darkness by wrapping the container with aluminum foil until its use. The sample spends another 3 days at 37°C and 80 rpm in the shaker (MaxQ 4000 Benchtop Orbital Shaker, Thermo Scientific) extensible to 4 if the tissue is not cleared enough. R1 is the reagent that makes the sample transparent.

The next step would be to carry out the immunohistochemistry, before we incubate the sample with the primary antibodies is necessary to wash it with PBS 1x on a table shaker for 1 hour repeating the process 3 times. Once the chemicals from the R1 are withdrawn the IHC is performed following the steps explained in the previous section. The complete IHC last for 6 days, changing at the third day from primary antibodies to secondary antibodies and previously washing the tissue with PBT 0.1%. If a prolongation of the IHC protocol is needed then it would better to add a day of incubation with the primary antibodies to prevent the secondary antibodies from binding molecules that are not of our interest and therefore obtaining a false positive that would produce noise in the image later. When the IHC is finalized the samples are washed again with PBT 0.1%, 1 hour of duration repeated 3 times. Finally, the incubation of the samples with R2 takes place, leaving the specimens 1 day at 37 °C and 80 rpm. Once they have completed the clearing process the samples are ready to be visualized. They can be used immediately or stored at 4 °C in the cold chamber.

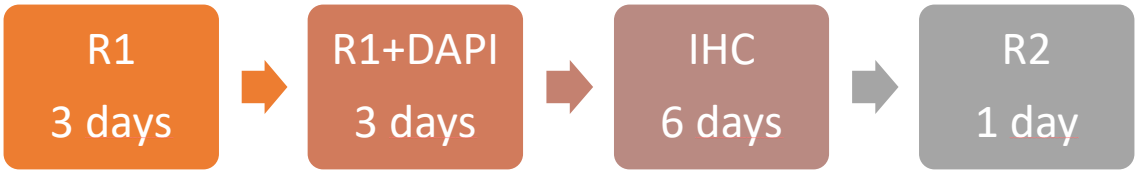

Figure 15. Periods for CUBIC protocol in mouse brain.

2.5 Sample preparation

The samples must be placed in R2 inside a glass tube that is placed in the platform of the SPIM. With the help of the micrometric motors of the SPIM the platform is moved and positioned assuring that the tube with the sample of interest is immersed in the glass cuvette filled with immersion oil (Johnson Baby Oil) at the intersection of the excitation and detection paths.

But the act of placing the sample to obtain a suitable view and preparing the tissue for taking the image is the most important part and has its difficulties. The sample

floats in the R2 staying really close to the limit of the liquid where the lasers are disrupted and artifacts are caused. It can also change its position from one study to the next one.

One possibility for avoiding this disadvantage is embedding the sample in a special agarose, the Low Melting agarose that is dehydrated and the SPIM's lasers can go through it. With the help of a syringe with the tip cut we are going to embed the sample in a perfect cylinder of agarose and then we are going to dehydrate the agarose in order to remove the water.

Embedding Protocol

The low melting agarose is prepared at a concentration of 1.5% with distilled water. Once it is ready it is heated in the microwave and poured inside the syringe. The samples are placed in the inside trying to leave them in a position that allow as to take images in the view that better suits each case (sagittal, axial or coronal) later while the agarose is cooling down. Once it has solidified and we have the cylinder of agarose with the sample inside we are going to dehydrate the agarose by placing it in a mixture of PBS 1x and sucrose at 20% during 30 minutes at 37 °C and 80 rpm in the shaker. Then the indexes of refraction need to be homogenized again and the cylinder is immersed in R2 again overnight at the same conditions as before. It is not necessary to reintroduced and incubate again the sample with R1 since we have just added agarose that has no lipids. Now they are ready to be visualized.

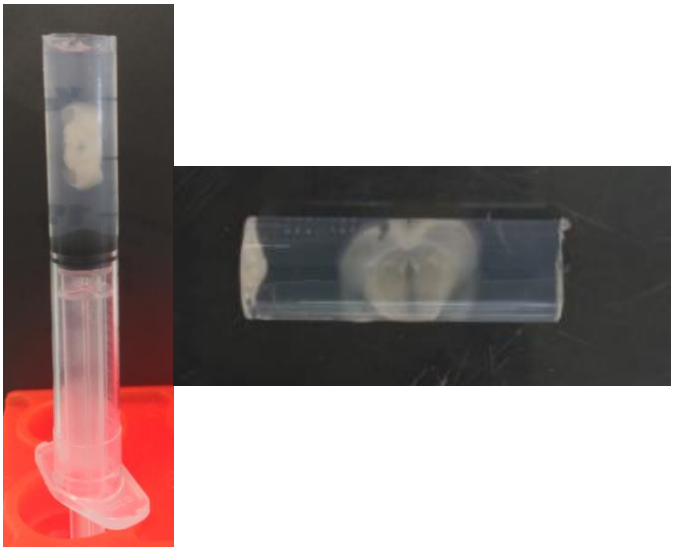

Figure 16. Sample preparation for the visualization in SPIM. Agarose cylinder with a mouse brain coronal section.

2.6 Image acquisition

2.6.1 SPIM microscopy

Single Plane Illumination Microscopy was used to acquire 3D images of the brains of mice. It was used to find the three-dimensional location of the GFP protein in the case of the mice and to visualize the vasculature.

The SPIM used was a custom-made microscopy design and built by Dr. Jorge Ripoll of University Carlos III of Madrid and placed at the Hospital Gregorio Marañón in Madrid at the Medical Imaging Laboratory.

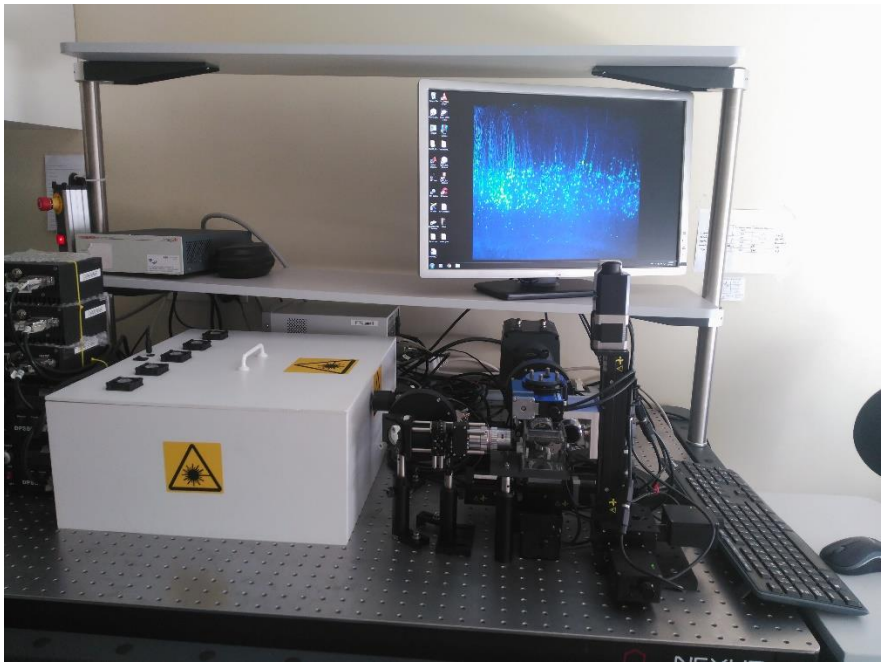

Figure 17. Homemade SPIM in Hospital Gregorio Marañón.

This SPIM in particular is formed by five different excitation lasers with different wavelengths and a selection of emission filters.

Excitation laser λ (nm)	Emission filter λ/bandwidth (nm)	Matching Spectra Fluorophores or Dyes
405	475/50	DAPI dye
473	531/40	Alexa Fluor 488, GFP

532	607/70	Alexa Fluor 555, Cy3 dye
590	624/40	Alexa Fluor 594, mCherry
635	670/10	Alexa Fluor 647, Cy5 dye

Two of these lasers, the 532 and 590, have wavelengths that overlap and they cannot be used together, therefore for all practical purposes only four lasers can be used at the same time, allowing us to study and label 4 molecules at once, using the 405 channel always for the DAPI.

There are two magnification objectives available to take images, the 2x and the 5x detection objectives. First of all, the 2x objective is used to make a general image and locate the region of interest and the 5x is used after that to focus on the region of interest being able to appreciate more details. In the following table, we have the data of some of the properties of the two different objectives, the numerical aperture (NA) which is a measure of its ability to gather light and resolve fine specimen detail at a fixed object distance, the working distance (WD) that is defined as the distance from the front lens element of the objective to the closest surface of the coverslip when the specimen is in sharp focus, and the depth of field (DF), defined as the axial range through which an objective can be focused without any appreciable change in image sharpness.

	NA	WD (mm)	DF (μm)
2x	0.055	34	91
5x	0.42	34	14

As we have seen in the previous chapter the direction of illumination, which is the one that the lasers follow exciting the sample, and the direction of detection, where the detection objectives that capture the fluorescence emitted by the sample after being excited, are located in orthogonal paths. In the intersection of these two paths a cuvette is found, filled with immersion oil and with the help of some motors a tube with a sample immersed in R2 inside and attached to a platform is placed in this exact point. The configuration of the homemade SPIM available at Hospital Gregorio Marañón is represented in the following figure:

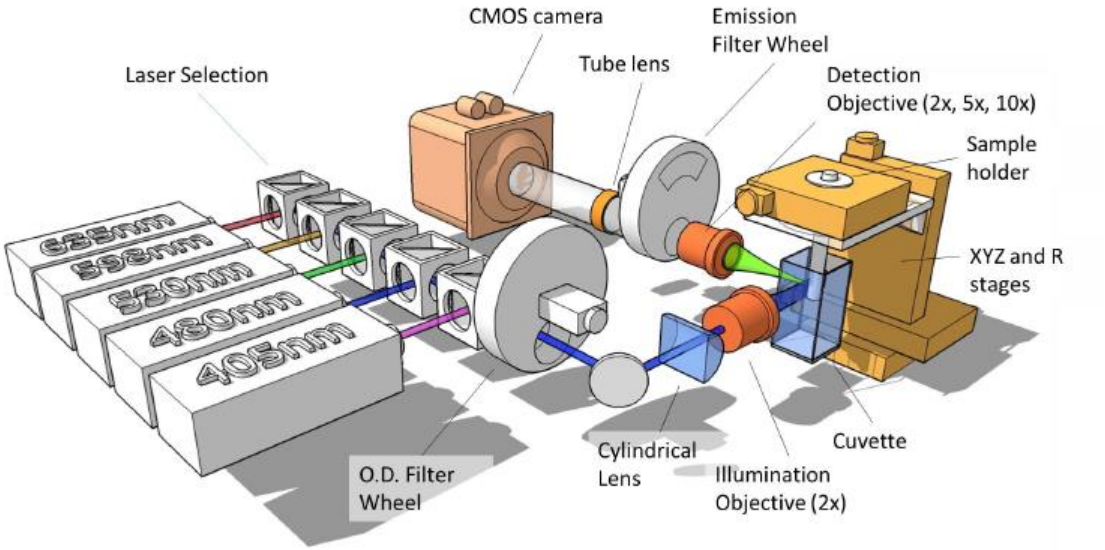

Figure 18. Physical distribution of the components of the SPIM microscope at Hospital Gregorio Marañón [28].

The SPIM software was also provided by Dr. Jorge Ripoll who developed it by himself. It is called Opt3D Experiment and it allows to take the image and control all the settings. The user manually places the specimen in the field of view (FOV) of the objective lenses with the help of the motors that control the platform and move it in the x, y and z directions as well as rotating the sample and refocusing the detection objectives.

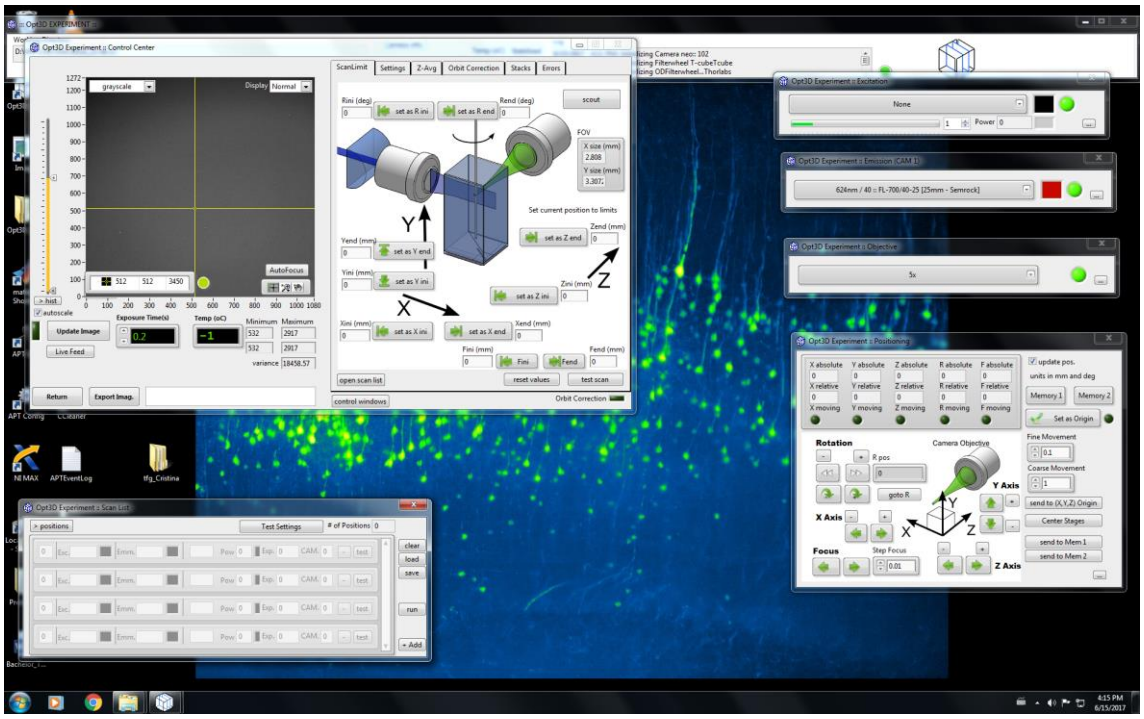

Figure 19. Opt3D Experiment interfaz

With the help of the software the area of interest is chosen and it allows programming the sequence of excitation lasers together with its respective emission filters and changing some important parameters for the obtention of the desire image. It also displays a screen where you can see the sample in live time.

The parameters that can be chosen by the program are:

- The magnification objectives
- The excitation lasers and the power of excitation
- The emission filters that correspond to each excitation laser
- The initial and final points in the z direction, the direction in which the sample is going to move.
- The camera exposure time
- The distance that the sample is going to move between two consecutives images of the 3D stack.

The SPIM have a powerful scientific CMOS camera, Neo 5.5 sCMOS camera (Andor Corp, Belfast, United Kingdom) that is thermoelectrically cooled to reduce electronic noise. The detector size is 6.5 µm x 6.5 µm and the sensor of the camera has 2560 x 2160 active pixels.

The image resolution is given by several factors, such as the camera pixel size, the magnification objective that we are using and the binning. The binning allows the combination of charges from adjacent pixels and this can offer benefits in faster readout speeds and improved signal to noise ratios although at the expense of reduced spatial resolution.

The formula for the image pixel size would be:

$$\textbf{Image Pixel Size} = \frac{\textbf{Binning} \times \textbf{Camera Pixel Size}}{\textbf{Magnification Objective}}$$

Binning of two adjacent pixels was used during the experiments so the final image size was 1280 x 1080 pixels

2.6.2 Confocal microscopy

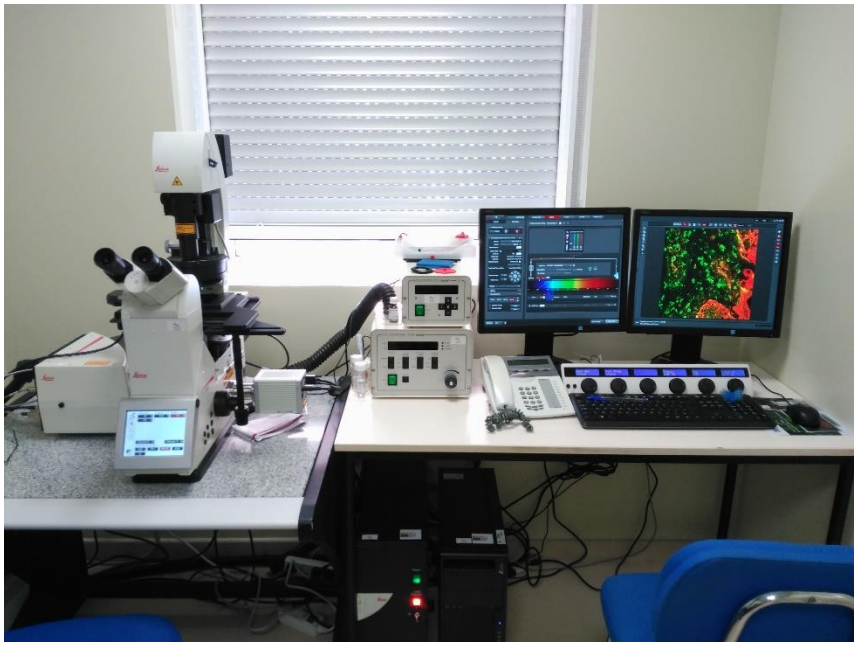

Figure 20. Confocal microscope at Hospital Gregorio Marañón.

The images acquired with the confocal microscope were taken using the Leica TCS SPE Confocal Microscope model with 10x and 20x objectives. It was used with the thick slices

of mice brain that were prefunded with the lectin to see the position of the vasculature with respect the location of the neuronal stem cells marked with Sox2 antibody.

Using the confocal microscope images with higher resolution than with the SPIM can be taken because the objectives available are much more powerful and give more details but on the other hand the samples need to be sliced in sections of 100-500 μm if they are cleared, being 100 μm the maximum if they are opaque.

2.7 Image processing

Once the images have been taken they need to be process in order to eliminate artifacts that usually take place when using the SPIM. Some of these frequent artifacts are: the existence of stripes in the image, caused by air bubbles that are place inside the sample when the cleared protocol is performed or other obstacles such as dust, or pieces of tissue therefore the laser sheet collide with these obstacles, scattering is produced, the light does not penetrate through it , and from the point where the obstacles are a black stripe appears; dark lines that are caused when the fluorophores of the sample are bleached after several images or if the power intensity of the excitation laser was too high; the illumination artifact, which consists in a decay of the illumination as the light sheet penetrate into the sample. As we have seen the sample is illuminated from one side and the laser sheet has to travel through the sample, but if it is not well cleared or if it is too big the laser sheet is going to be losing power as the distance that it has to travel increases due to light absorption and scattering, leaving differences of illumination in the image between one side of the sample and the other one. These problems are solved by implementing some algorithms and using some software created by the Dr. Jorge Ripoll.

The stripes artifact is solved applying the “rolling ball” algorithm described in more detail in [29] developed in Matlab©. It consists mainly in morphological operations being the principals a closing operation followed by an opening operation. The closing operation would be the equivalent of a maximum and a minimum filtering. This operation is supposed to close all holes in the intensity graph and smoothes the graph to the background level but it also shifts edges. The reverse step was performed to undo this

shifting, which would be achieved by performing the minimum filter. The effect of the minimum filtering would be a smoothing of the holes and a refilling with the value of the background. The opening followed by a closing operation is what is known as rolling ball algorithm. The result would depend on the size of the structuring element used, the stripes are removed but it also implies a reduction of the details of the image.

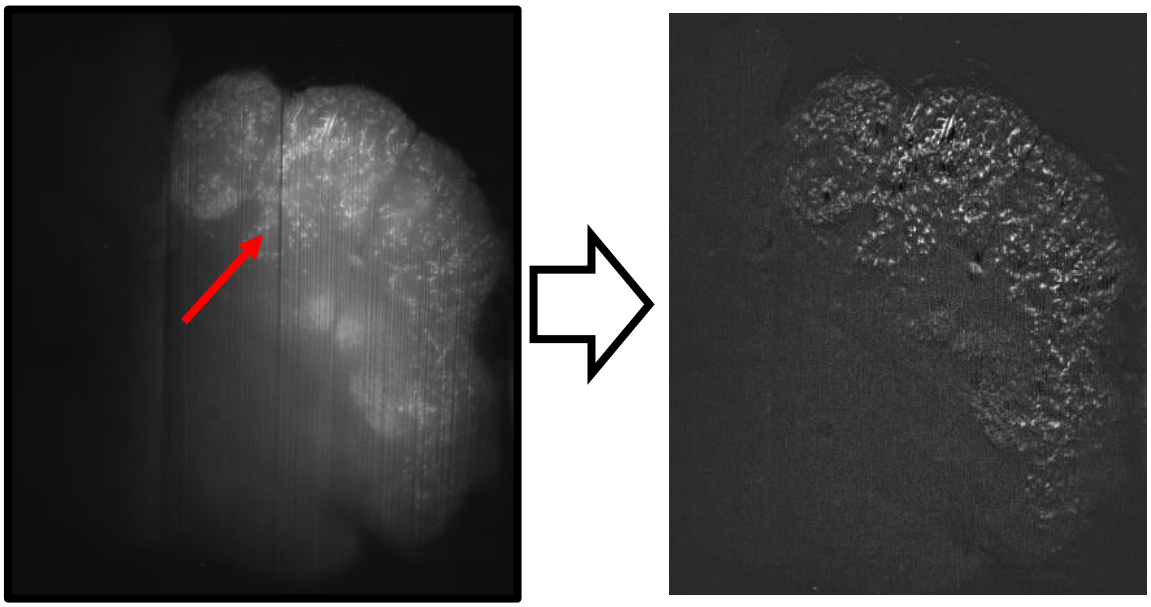

Figure 21.Stripes correction.

The illumination correction can be solved rotating the sample 180° and acquiring again the same thickness, then by reversing the order of the second stack of images a stitching would be performed with the help of a program, which would consist on an intensity-based registration that would join the two images in one matching up the common features. This stitching can be performed in the x and y plane.

Another artifact was stack shifting that was more obvious at the time of making the composite of the different channels, which consists on assigning each channel a color and assembling all together in the same image to see the distribution relative to each other. Sometimes, due to the misalignment of the lasers or that the micrometric motor, when going back to the initial position after finishing with one laser and starting with the next one, did not start in the same precise point, the channels did not match when making the composite, which had to be fixed with one of the software available. This fixation consists on the manual registration of the different channels, taking one of the images as a reference and trying to match some recognizable features of the images

from other channels with the reference one. This problem was solved, but results could be improved because the fact it is adjusted manually give rise to errors due to the inaccuracy of the human eye.

3. Results and discussion

The Medical Imaging Laboratory has been developing different techniques to clear biological tissues in order to work with optical microscopies. Among the techniques used the CUBIC clearing method was the chosen one, because it fulfills all the requirements. It is relatively fast, it reaches high degree of transparency, is cheap to reproduce, it is compatible with the IHC preserving the fluorophores and the anatomical structures, which was a very important condition for working with fluorescence microscopes.

The transgenic mice were breed in the University Complutense of Madrid, Veterinary Faculty and facilitated to our laboratory to clear, take images and process the images.

Mice brains were extracted after the perfusing transcardially the mice with PFA 4%. These brains belonged to mice at different stages of development, both embryonic and postnatal stages.

The objective of the study is to see the expression of the P2X7 receptor in the whole brain without sectioning to image large depths of tissue in a fast way avoiding photobleaching and the SPIM microscope is a good choice. As it is known from other experiments with the confocal microscope at depths larger than 100 µm light scattering became a problem and even if the samples are cleared, the maximum penetration would be 500 µm, even though it is already an advance and very time saving, because slices of 500 µm are easily sectioned with the vibratome avoiding the need of sample freezing or paraffin embedding.

After the brains extraction, they were cleared optimizing the periods of incubation with the different reagents depending on the size and age of the brains, and optimizing the protocol for embryo brains and postnatal brains. It has been proved that embryonic tissues are easier of make transparent than adult tissues, due to the fact that the complexity of tissues is different, being more complex as the brain is more mature [27].

Also, the size is important and, the clearing reagents needs longer to penetrate inside of the brain when the whole organ is cleared. This is one of the reasons why the brain was divided in two hemispheres. Therefore, the time of incubation with R1 had to be carefully controlled when embryonic tissue is cleared, on the grounds that if the sample is too much time submerged in this reagent, the tissues can be disaggregated. When tissue degrades in such a manner the physical and molecular structure is lost and the samples become useless for the study.

On the other hand, if the time of incubation in R1 is insufficient the tissues would not be well cleared and when taking images with the SPIM the laser would not penetrate through the whole sample and the resolution of the image will be lower.

Finally, it was determined that the times of incubation for small, embryonic tissue were:

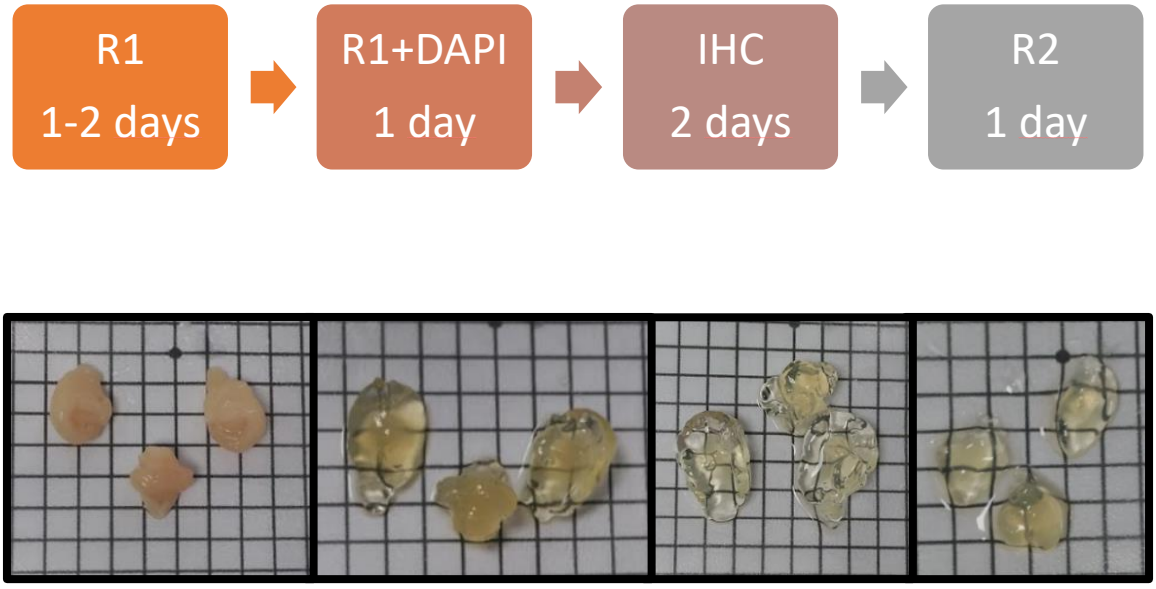

Figure 22. Evolution of tissue clearing process using the CUBIC protocol when clearing mouse brain.

During the clearing process sometimes, the immunohistochemistry was performed to enhance the fluorescent power of the transgenic brains. The principle of the CUBIC method is the lipids removal from the tissue to make it transparent. The IHC can be performed after R1 incubation followed by R2 incubation to match the refraction indexes again. This is useful, for example, to enhance endogenous fluorescence like the GFP. Also, IHC was perform with the thick slices of brains prefunded with lectin to see the microvasculature in the brain.

After R1 has removed the lipids the sample is already transparent, but when the IHC is performed, it has to be washed with PBS 1x first, which makes the sample opaque again due to the change in the refraction index. When the IHC is finished the sample is incubated in R2 that does not clear more than the R1, it only modifies the index of refraction at 1.48-1.49 and it becomes transparent again.

This technique has some difficulties, presenting throughout the process of mouse perfusion, sample slicing and tissue clearing.

During the developing of the project the evolution of the structures can be followed from an E13 stage (134 days after the moment of the fertilization, embryonic) to a P12 (12 days after the birth, postnatal). Here is a list of the stages that were cleared and analyzed:

Stage	Whole/Divided
E13	Whole
E14	Whole
E18	Whole
P0	Whole
P7	Hemispheres
P9	Sliced (Spinal Cord)
P12	Hemispheres

After the tissues are cleared is the images are acquired with the SPIM microscope. The embryonic and P0 brains were small enough to fit in the crystal tubes but the bigger ones (postnatal brains) were divided in three parts, both hemispheres and cerebellum, to fit them inside the tube and to get better images, as explained in previous chapters.

Images were acquired using the 2x objective; DAPI staining was used to locate the areas of interest in the Live screen when working with the SPIM microscope avoiding the fluorescence quenching in the process of placing the sample in a proper angle.

Once the sample was focused in the area of interest, the lasers that are going to be used in the study were selected, modifying the intensity power to avoid, if possible, saturating areas of interest. In a post processing work, poor illuminated pixels can be seen by doing some modifications, but the saturated pixels cannot be changed.

The two main lasers used were the ones with the wavelengths of 473 nm and 405 nm, for the GFP and the DAPI dye respectively. Once the sample was excited with these two lasers, the fluorescence emitted in two different wavelengths: 531 and 475. The camera took images every 20 microns sometimes capturing even more than 4 mm of depth.

After acquiring the images, they were processed by changing the look up tables to be able to see each channel in a different color at the same time and making a composite of them, matching the channels between them. There are artifacts inherent to the SPIM microscopy that need to be removed like the removal of the stripes that appear when there is an obstacle in the path of the light sheet and the light is unable to pass through it. These obstacles can be dust, bubbles, edges, etc. and they generate black stripes in the direction of illumination. Whenever it is possible the stripes are removed but sometimes when removing the stripes important information is lost and a balance has to be reached. Also changes in the bright and contrast of the images were needed to improve them in a proper manner. These minor processing steps that improve the image were performed with FIJI program. FIJI is an image processing package, distributed by ImageJ that is free, open source and has a lot of plugins that facilitate scientific image analysis.

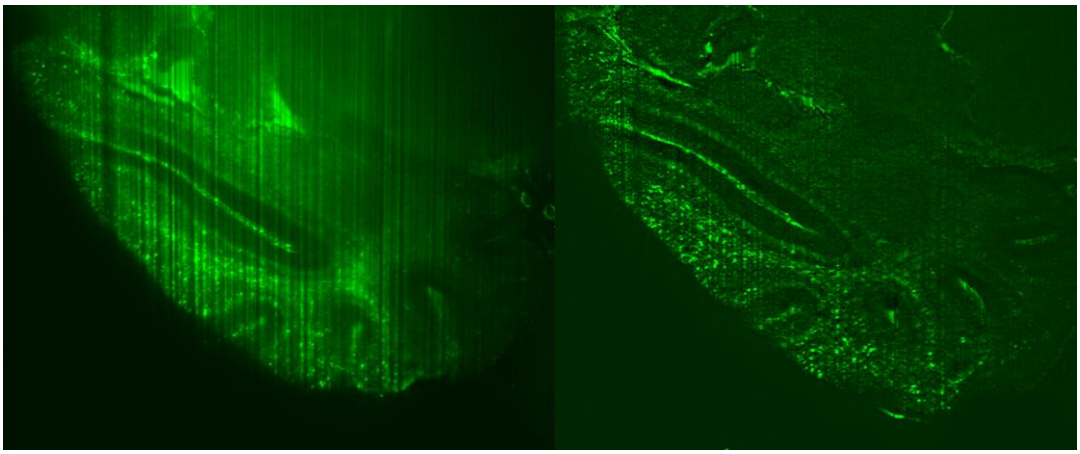

Figure 23.Stripes correction of a cerebellum image belonging to a P7 acquired with SPIM microscopy.

In the following figure (Fig. 24) different mouse developmental stages from E13, the earliest stage that has been studied to P12, were the brain is fully formed. The evolution of the brain during the embryonic stages is more appreciated, as might be expected, than in the postnatal stages, however the cerebellum is not developed until the mouse is borned, that is the reason why the embryonic stages doesn't have cerebellum even though an increasing in the amount of receptors in the caudal area of the brain is perceived.

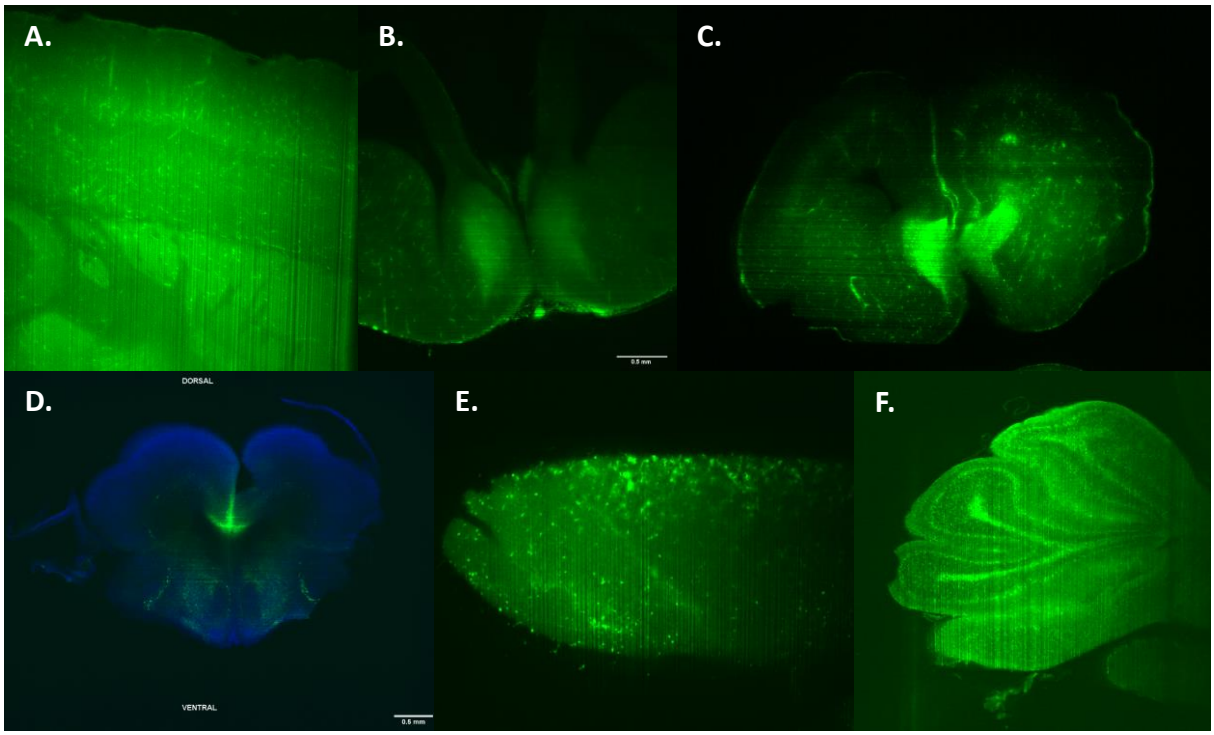

Figure 24. Expression of GFP - P2X7 receptors in a mouse brain. A: Image of the cortex of an E13 stage. B: Coronal view of E14 stage that express fluorescence in the thalamus zone. C: Coronal view of an E18 with a more developed thalamic zone. D: Coronal view

of a P0 stage in the caudal area. E: Expression of the receptors in the cortex of a P7 brain. F: Cerebellum of a P12 stage.

Observing these images an idea of the distribution of the purinergic receptors can be estimated, the following scheme (Fig. 25) can be helpful to know in which area of the brain these images can be found.

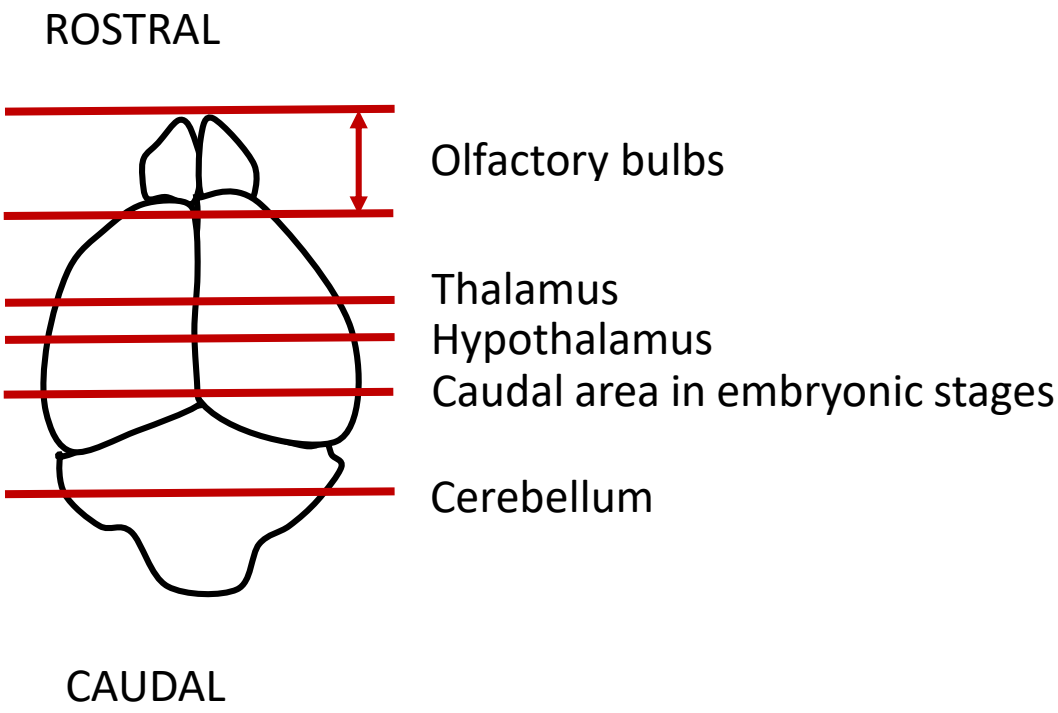

Figure 25. Anatomical reference of images acquired.

Following this outline, the images from Figure 24 would be place on one of those areas, excluding the cortex that covers the whole brain and can be found in any area. The image belonging to a E14 stage shows how the thalamus start to form and the presence of the receptors in it, two holes can be appreciated which would be the lateral ventricles. A great change from the E14 to the E18 is perceived, the thalamus zone is much more developed as well as the septal nucleus and the fluorescence perceived by the CMOS camera has increased also. In the image D, a coronal view of the caudal part of an E18 brain is shown. In this stage, the cerebellum is not developed yet but an increase of the fluorescence in the posterior part is witnessed. In the P7 stage the brain is fully grown and the image of the cortex presents a lot of fluorescence comparing it with the embryonic stage. And finally, the cerebellum belonging to a P12 stage shows plenty of fluorescence in the cerebellar loops, which indicates that the cerebellum is an area rich in purinergic receptors.

Analyzing these images, an increase in the expression of fluorescence is observed, which means an augmentation of the number of P2X7 receptors, is appreciated during the

evolution of the specimen especially in the olfactory bulbs, thalamic area, the hypothalamus, and in the cerebellum once it has grown.

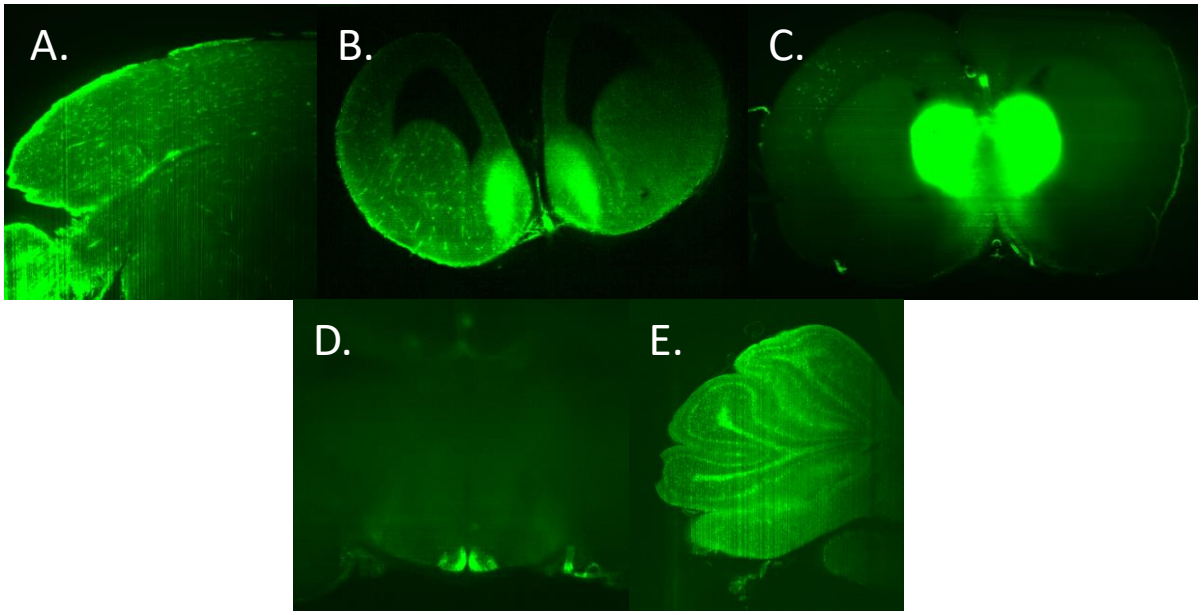

Figure 26. Main areas were P2X7 receptors are expressed. A: Cortex P12. B: Thalamus E14. C: Thalamus and cortex E18. D: Hypothalamus P0. E: Cerebellum P12.

The purinergic receptors are well defined in these areas, except the ones that are present in the cortex, that are distributed in a larger area, that cannot be delimited, but it is observed to be an increase of the cortical receptors in the most rostral are, that is near the olfactory bulbs. On the other hand, the cerebellum is one of the richest areas, and as we have seen before the moment of the birth the cerebellum is not developed yet, but in the area that is going to give rise to these part of the brain the accumulation of receptors is a patent fact. It can be seen in the following image (Fig.27). Following this study of the cerebellum some slices of spinal cord were analyzed with the confocal microscope to see the expression of the purinergic receptors in other area of the nervous system (Fig. 27).

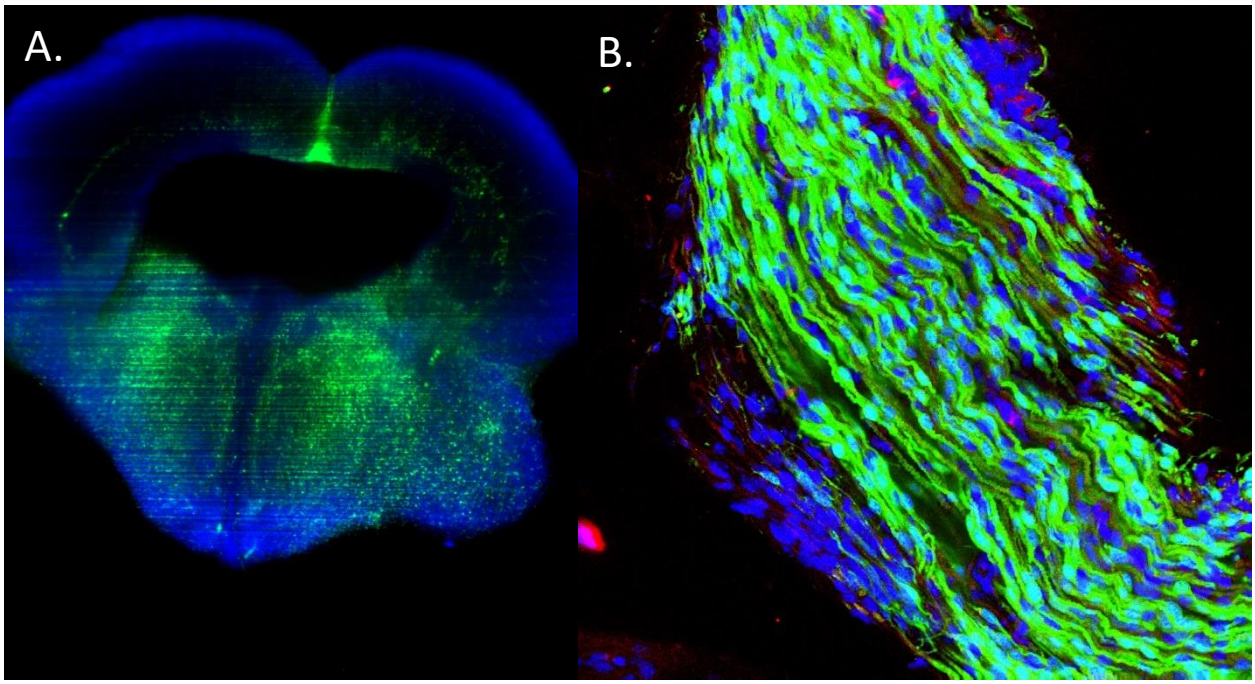

Figure 27. A: Expression of P2X7 receptor in the formation of the cerebellum, P0 stage. B: Expression of P2X7 receptors in a spinal cord slice acquired with the confocal microscope, P9 stage.

Another important tool used to locate and make an idea about the anatomical distribution of the labelled receptors are the volume renderings. Starting from the 2D stack of images and being sure to enter the correct properties of the images, such as the measure of the pixel and the distance between two consecutive images, a 3D projection of the 2D stack can be achieved. This projection allows to see all the stack ordered with the same measures than in the reality which reconstructs the reality producing a rendering. Apart from that, ImageJ has some plugins that can make volumetric 3D reconstructions from the stack of 2D images captured by the SPIM, as an opaque solid. Also, a plugin special for tubular forms that is helpful to reconstruct the vasculature of the lectin- perfused brains.

Other programs as 3D Slicer are able to make a reconstruction of the location of GFP protein and obtain a volume where the purinergic receptors are concentrated. This program is very helpful because it has a lot of tools and can reconstruct 3D models very easily allowing the user to play with the different views of the stack: axial, coronal and sagittal.

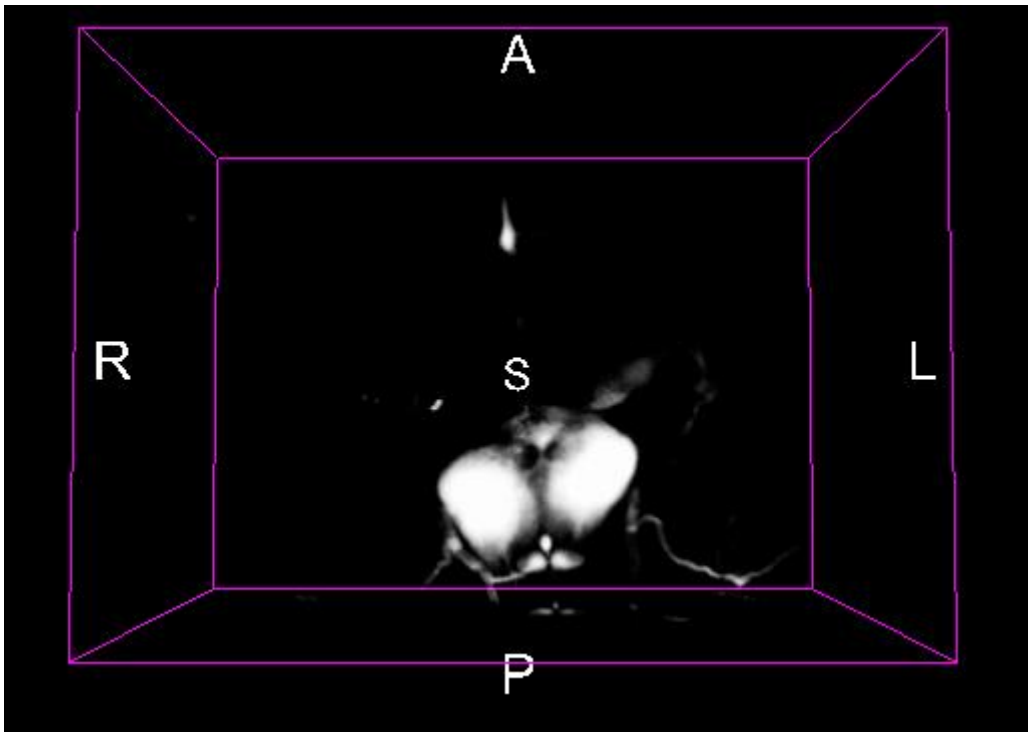

Figure 28. 3D reconstruction of GFP expression inside an E18 mouse brain.

As we can see in the previous figure the GFP expression is located mainly in two big "balls" in the middle of the ventricles, in the thalamic and hypothalamic zone and also in the most caudal part of the brain. But also, some vessels are present in the image due to the autofluorescence they emitted mainly when the animals are not well perfused.

An on-going work is to try to eliminate this vessels from the images, because it is not very esthetic, by exciting with the green laser and acquiring not only with the emission filter needed to capture the GFP fluorescence but also with other emission filters to capture the autofluorescence. Then the solution would be to subtract the two images to eliminate what is common to both of them, the fluorescence emitted by the vessels. After the subtraction, an advance image processing work should take place.

Consequently, when the vasculature was found the idea of seeing how it is related with the purinergic receptors emerged. The objective would be to visualize the vessels in the transgenic model, but as they are expensive to produce, the procedure followed was to develop the technique and practice in wild type mice to control all the parameters that will affect the correct perfusion, for example the time of incubation, the force of the peristaltic pump, etc. It was contemplated the relation with the vasculature and the neuronal stem cells too. To visualize the vasculature completely and not also the badly-

perfused vessels a new approach was tested. This new approach consisted on perfusing the mice with lectin from the tomato.

During the perfusion FITC- Lectin was introduced in the circulatory system of the mouse with the help of the peristaltic pump and it was incubated during 2 minutes. From this moment, the extracted brain would have to be in darkness, carried in tubes with aluminum foil recovering them.

The clearing method was performed normally and in the IHC the antibody Sox2 was used to label the stem cells. When the samples were ready to take images, the confocal microscope was chosen because of its powerful objectives and because the brain had been sliced to search the regions of interest were most of the neuronal stem cells are located, the svz and the dg.

Images were taken with the 20x detection objective and it was observed that the lectin was successfully perfused and the Sox2 has worked.

From these 2D stacks it was possible to make a three-dimensional reconstruction. First, the composite was made, where the two channels were put together and then a 3D projection was performed to reconstruct the vasculature. Once we had the projection a volume rendering video was made where it can be observed the relation of the vasculature and the stem cells in the space, even though the antibodies that label the stem cells have not penetrated the entire coronal section, only half of it, which can be solve by incubating the sample with primary and secondary antibodies for longer periods of time. In the following figure, we can see these steps and we can observe that the stem cell niche is located near the larger vessels, which is an interesting fact to study for future research projects.

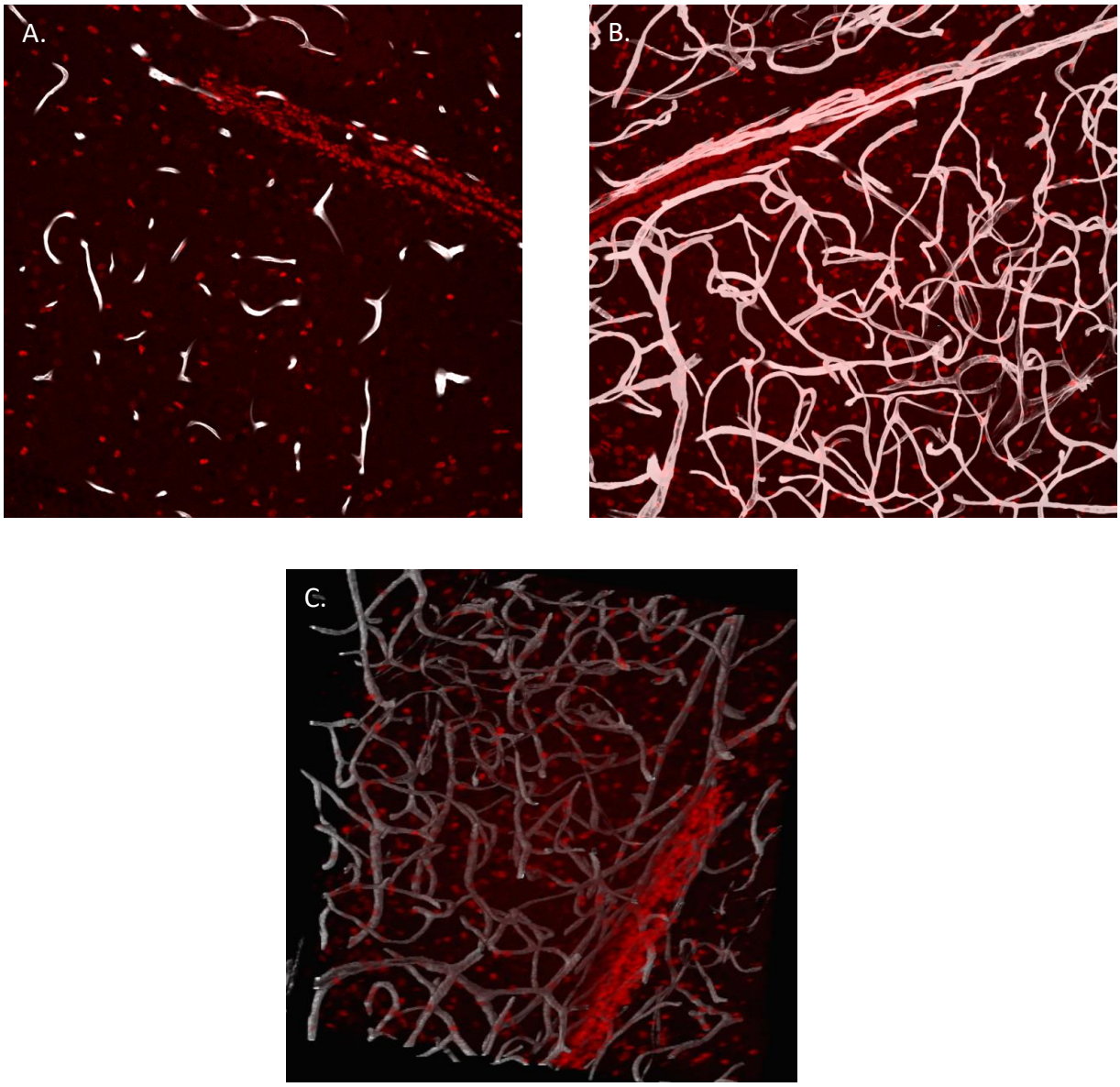

Figure 29. A: Composite where the vasculature and the niche of stem cells are present. B: 3D projection. C: 3D volume rendering where the location of vasculature labelled with lectin can be seen respect the stem cells marked with Sox2.

4. Conclusions

The CUBIC tissue clearing method has been optimized for both embryonic and postnatal stages, adjusting the periods of incubation in R1 and R2. For embryonic brains, the incubation periods were reduced to ensure the integrity of the tissues once they are cleared and, for postnatal brains the periods were longer to guarantee the clearing of the whole organ.

Immunohistochemistry technique was performed in the brains to enhance the endogenous fluorescence of the transgenic mice.

Images from the samples were acquired with SPIM microscopy to visualize the anatomical location of the GFP-P2X7 receptor in the brain. In the E13 brains, the purinergic receptors were visible in the cortex. In the E14 brains, the presence of the receptors was distributed by the cortex and the emerging thalamus zone. The E18 brains have a more developed thalamic zone were the P2X7 receptors are located, as well as in the cortex, the hypothalamus and in the most caudal part of the brain were the cerebellum will be formed once the mouse is borned. The postnatal stage P0 is similar to the E18 but with the ventricles and thalamic area fully grown and it shows fluorescence in the same areas than E18 brains. The rest of the postnatal stages studied in this project, the P7 and P12, both looked very similar presenting fluorescence in the cortex, thalamic and hypothalamic zones and in the cerebellum that was not present in any of the previous stages. In the cerebellum, the P2X7 receptors are very abundant, and it was also analyzed a sample from the Spinal cord using the confocal microscopy which showed plenty of the GFP protein.

Apart from locating the receptors in the brain, a protocol for visualizing vasculature was successfully optimized in wild type mice, in order to use it with the transgenic mice in future works.

5. Project costs

The costs for this project were approximated taking into account three types of resources: human resources, laboratory material and technical equipment.

Human Resources Costs

Human resources costs comprehend the salaries of the team members of the project.

Human resources	Cost/Hour	Working Hours	Total Cost
Project Manager/PI	€35	400	€14,000
Student	€25	670	€16,000
			€30,000

Technical Equipment Costs

Technical equipment costs include laboratory machinery and the different software and the computer hardware used. Other laboratory equipment includes small everyday use instruments such as the magnetic stirrer, the precision weighting balance, the Vortex mixer, etc.

Equipment	**Average life time in years**	**Unit cost**	**Units**	**Depreciation per month**	**Months employed**	**Total cost**
SPIM	10	€120,000	1	€ 1,000.0	2	€2,000.00
SPIM software license	1	€1,000	1	€83.33	2	€166.66
Image J software		€0	1	€0.00	9	€0.00
Matlab software license	1	€2,000	1	€166.66	9	€1,499.99
Office 2010 license	2	€139	1	€5.80	9	€52.2
Personal computer	5	€700	1	€10.00	9	€104.99
Shaker	5	€5,000	1	€83.33	9	€749.97
Other laboratory devices	5	€4,500	1	€75.00	9	€883.00
						€5,456.81

Laboratory material costs

Costs of laboratory material includes all reagents and immunohistochemistry antibodies, as well as expendable material. Expendable laboratory material includes everyday items used such as Falcon tubes, Pasteur pipettes, multiwall culture plates, parafilm, etc. This material is supplied directly at the Hospital and during the project we acquired eight packs containing different items, as needed. The approximate cost of each order was 30€.

Laboratory Material	**Units**	**Cost/Unit**	**Total Cost**
Clearing Reagents	1	€400	€400
Laboratory Reagents	1	€500	€500
IHC Primary Antibodies	3	€250	€750
IHC Secondary Antibodies	5	€200	€1,000
Expendable laboratory material	8	€30	€240
			€2,890

SAIS associated costs

SAIS (Servicios de Apoyo a la Investigación Sanitaria) facilites are provided by the Health Research Institute of Hospital General Universitario Gregorio Marañón to biomedical research laboratories as low-cost services. Costs derived from SAIS facilities are shown in Table 8.

SAIS facilities	**Cost/Hour**	**Hours employed**	**Total Cost**
Confocal Microscopy	€10	44	€440
			€440

6. Appendix

Recipe of the different reagents used in the different solutions performed in the laboratory are given in the following section.

6.1 PBS 10X (1L)

- 80.06 g (1.37M) of NaCl
- 1.12 g (15mM) of KCl
- 11.36 g (80 mM) of Na2HPO4
- 3.67 g (27mM) of KH2PO4

Add distilled water up to 1 L. Let it dissolve on a magnetic mixer with a stirring beat.

6.2 PBS 1x (1L)

Add 100 mL of 10X PBS with 900 mL of distilled water. Check the pH and adjust it to pH 7.4 with NaOH (if it is below 7.4) or HCL (if it is above 7.4).

6.3 PFA 4% (1L)

Add 800 mL of PBS 1X to a glass beaker on a stirring plate. Heat up to 55°C while stirring. Add 40 g of PFA powder to the heated PBS solution. This should be done in a ventilated hood since PFA is toxic. Add a few drops of NaOH with a pipette to support dissolving. Adjust the volume of the solution to 1 L with PBS 1X. Once the PFA is dissolved, readjust the pH to 7.4 by adding drops of HCl. PFA should be prepared right before the perfusion.

6.4 Reagent 1 preparation (10 ml)

Add 3.5 mL of distilled water, 2.5 g of urea and 2.5 g of N,N,Nâ,Nâtetrakis(2-hydroxy-propyl)ethylenediamine to a glass beaker. This chemical is highly viscous, so it can be diluted down 1:4 with water. Dissolve the mixture by stirring. This solution does not need to be heated. Add 1.5 mL of Triton 100X and stir. Degas the solution with a vacuum pump until air bubbles raised to the surface.

6.5 Reagent 2 preparation (10 ml)

Add 1.5 mL of distilled water and 5 g of Sucrose to a glass beaker. Let it dissolve on a heated stirrer. Increase the temperature to almost 100°C. Avoid overheating of the solution. It will dissolve in approximately 30 minutes. When it is almost dissolved, decrease the temperature to 30 °C but keep stirring. Add 2.5 g of urea and let it dissolve on a non-heated stirrer in order to prevent the decomposition of the urea. Add 1g of Triethanolamine and stir. Adjust the weight constantly with distilled water. Degas the reagent with a vacuum pump as done for CUBIC-reagent 1. This solution is highly viscous. Immersing the Falcon tube in warm water during the degasification will improve the speed.

6.6 Primary Antibody Solution (20 mL)

Add 0.1 g of Bovine Serum Albumin (BSA) and 0.002 g of Sodium Azide to 20 mL of PBT 0.1%. The solution can be aliquoted in small quantities and stored at -20°C until used.

6.7 Secondary Antibody Solution (20 mL)

Add 0.02 g of Bovine Serum Albumin (BSA) and 0.002 g of Sodium Azide to 20 mL of PBT 0.1%. The solution can be aliquoted in small quantities and stored at -20°C until used.

6.8 PBT 0.1% (1L)

Add 1 mL of Triton 100x to 1 L of 1X PBS.

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