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Påvisning av nevrogenese ved bruk av immunfluorescerende fargemetode

Detection of neurogenesis by immunofluorescent staining

Av / by

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Sammendrag

Om lag 50 millioner lider av Alzheimers sykdom (AS) på verdensbasis, og antallet er estimert å triple i løpet av år 2050. Alder er en av de største risikofaktorene for å utvikle AS. Ettersom levealderen øker og det per dags dato ikke finnes noen kur, er behovet for å finne behandling for nevrodegenerative sykdommer som AS stor. Det er fastslått at nydanning av nerveceller, også kalt nevrogenese, forekommer i hippocampus. Dette bidrar til å opprettholde hippocampus-avhengige kognitive ferdigheter. Nylige studier foreslår at eksponering av blodprodukter kan være en potensiell forebyggende behandling, ved å ha en mulig positiv innvirkning på nevrogenese i en aldrende rottehjerne.

En av forskningsgruppene på Cardiac Exercise Research Group (CERG) har et pågående prosjekt hvor friske, unge rotter gjennomgår et høy-intensitets treningsprogram over 6 uker. Videre blir blodet fra disse rottene injisert i en rottemodell som har utviklet et tidlig stadium av AS for å undersøke om nevrogenesen blir fremmet av behandlingen. Målet for prosjektet vårt var derfor å utvikle en tilpasset immunhistokjemisk protokoll for påvisning av nysyntetiserte nevroner, til bruk for laboratoriet på CERG.

Laboratoriearbeidet bestod av å snitte vev for deretter å velge ut snittene assosiert med hippocampus. Videre ble de utvalgte snittene farget med en metode basert på «BrdU assay for neurogenesis in rodents» av Wojtowicz & Kee, hentet fra Nature Protocols 2006 og «Immunofluorescent Protocol for BrdU» av Kitabayashi (2000), samt «BrdU staining and BrdU assay protocol» fra Abcam. Vi testet ulike parametere med målet om å optimalisere en protokoll tiltenkt bruk på laboratoriet til CERG.

Ingen av forsøkene på å optimalisere en BrdU protokoll resulterte i fluorescerende signal. Testresultatene ble derfor vurdert til negative.

Prosjektet hadde en begrenset tidsramme, og av denne grunn ble ikke BrdU protokollen fullstendig optimalisert. Det er derfor nødvendig med videre uttesting for å fullføre etableringen av den immunhistokjemiske fargemetoden med anti-BrdU for deteksjon av nysyntetiserte nevroner i en rottemodell for AS.

Abstract

At present, a global prevalence of Alzheimer´s disease (AD) is reported to be as high as 50 million. By the year of 2050, this number is projected to triple. The largest risk factor for developing AD is increasing age. As human lifespan increases, and since no cure currently exists, the need for finding treatments for neurodegenerative diseases such as AD is obvious. It is well established that hippocampal neurogenesis contributes to maintain hippocampus-dependent cognitive abilities. According to recent studies, potential AD preventative treatments include exposure of blood products (plasma), which possibly have a positive impact on neurogenesis in an aging rat brain.

One of the research groups at Cardiac Exercise Research Group (CERG) has an ongoing project, where blood from healthy, young rats that have undergone a 6-week high intensity exercise-programme is injected to rats with a model of early stage AD to investigate whether this promotes neurogenesis. The aim of our project is therefore to develop a customized immunohistochemistry protocol for detection of newly synthesized neurons to be used at the laboratory at CERG.

The laboratory work consisted of tissue sectioning and thereafter selecting sections associated with the region of interest, the hippocampal region. Furthermore, the sections were stained using methods based on a protocol adapted from “BrdU assay for neurogenesis in rodents” by Wojtowicz & Kee from Nature Protocols 2006, “Immunofluorescent Protocol for BrdU”, by Kitabayashi (2000) and Abcam´s “BrdU staining and BrdU assay protocol”. In our project different parameters were tested with the aim of successfully optimizing a protocol intended for use in the laboratory at CERG.

None of the attempts to optimize a Bromodeoxyuridine (BrdU) staining protocol for neurogenesis detection resulted in a specific nuclear immunofluorescence signal, and the test results were stated as negative.

The BrdU staining protocol was not entirely optimized, as this was not achievable within the timeframe given for the project work. Therefore, it is necessary to perform further tests to complete the establishment of an immunohistochemical staining method with anti-BrdU for detection of newly synthesized neurons in a rat model for AD.

Abbreviations

AD – Alzheimer's disease

Aβ – Amyloid beta

BrdU – Bromodeoxyuridine

CERG – Cardiac exercise research group

DMSO – Dimethyl sulfoxide

IF – Immunofluorescence

IHC – Immunohistochemistry

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1.0 Introduction

One of the research groups at CERG has an ongoing project, where blood from healthy and exercised trained rats is injected to rats with early stage AD to investigate whether this could potentially have a therapeutic effect for neurodegenerative diseases by increasing hippocampal neurogenesis. Neurogenesis can be observed using a BrdU staining method (1). For our study project, brain tissue were collected from the plasma-injected AD rats. To detect neurogenesis in the rat brain, a BrdU staining method was used. The aim of this thesis was to optimize a protocol for this IHC staining method, to present a protocol applicable to the research group at the specific laboratory at CERG. This particular staining method is widely used in laboratories worldwide, both in brain tissue as well as in other tissues. Each laboratory has to establish their own protocol, optimized for their specific work.

The image below (figure 1) illustrates the tissue of the rat brain from another study, with the fluorescence intensity we want to achieve in our study. The fluorescence used for this illustration is the same fluorescent secondary antibody that will be utilized in our study. In this specific image, the illuminating green colour shows plaque formation in the AD rat brain. To achieve a desired immunohistochemical staining result, we combined and adjusted a number of established protocols to customize an optimized protocol for CERG to use in prospective studies.

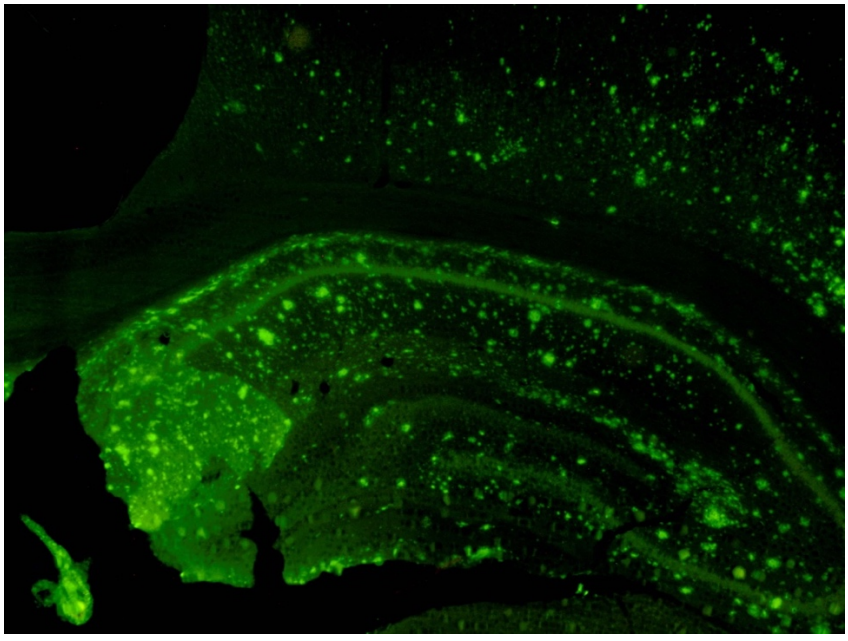

***Figure 1**: A photomicrograph showing positive immunofluorescence in the hippocampal region of the brain tissue, where plaques are visualized by fluorescent dots Provided by Kamilla & Lovise.*

1.1 Alzheimer's Disease

AD was first described in 1906 by the clinical psychiatrist and neuroanatomist Alois Alzheimer, as a “peculiar severe disease process of the cerebral cortex” (2). Today, AD is recognized as a progressive neurodegenerative disease and the most common form of dementia worldwide, accounting for an estimated 60-80 % of all dementia diagnoses (3). AD is characterized by progressive decline in two or more cognitive domains, including memory, language, executive and visuospatial function, personality and behaviour, causing loss of ability to carry out basic activities of daily living (4). The hallmark pathologies of AD include amyloid plaques primarily consisting amyloid beta (Aβ) peptide outside neurons, and neurofibrillary tangles formed by aggregated tau protein inside neurons. Eventually, these changes lead to the damage and death of neurons (3). Neurodegeneration with synaptic and neuronal loss leads to macroscopic atrophy. Regions in the brain affected in the early stages of AD, comprise both the hippocampal formation and the parahippocampal region (5, 6). In particular, the structure of the brain called the hippocampus tends to be especially vulnerable to damage in the early stages of AD. The hippocampal area is critical for both learning and memory, and an impairment of these abilities will significantly correlate with cognitive decline (7).

The risks associated with developing AD are multifactorial, however, the largest risk factor by far is advanced age. Due to the progressive aging of the population, AD represents one of the greatest medical challenges that face this century (8). At present, a global prevalence of AD is reported to be as high as 50 million and is projected to triple by the year of 2050 (9) (10). In the US, a new case of AD is currently recorded every 66 seconds. By 2050, a new case would be recorded every 33 seconds if no treatment is invented (9). To date there are no effective pharmacological nor non-pharmacologic therapy options for prevention and treatment of AD, as the current medicines do not inhibit the fatal outcome caused by damage and destruction of neurons (3, 11). This has resulted in a substantial need for optimized prevention, diagnostics and treatment of AD, that offer improved symptomatic benefit and disease-slowing capabilities (12). According to previous studies, one of the latest potential AD prevention and possible treatments include an exposure to blood products from young rats, which possibly has a positive impact on both synaptic plasticity and neurogenesis in the rat brain, an effect that is hopefully transferable to the human brain (13).

1.2 Neurogenesis in the adult hippocampus

The hippocampus is a part of the brain that plays an important role in both memory and spatial navigation. This structure is an extension of the temporal part of cerebral cortex, located in the medial temporal lobe of the brain. The hippocampus has been extensively studied, and prior publications imply that atrophy in particular in this region of the brain has great clinical consequences. Several neurological and psychological disorders severely affect the hippocampus, and this region is affected at an early stage of AD (14). The figure below illustrates a coronal section of the rat brain (figure 2).

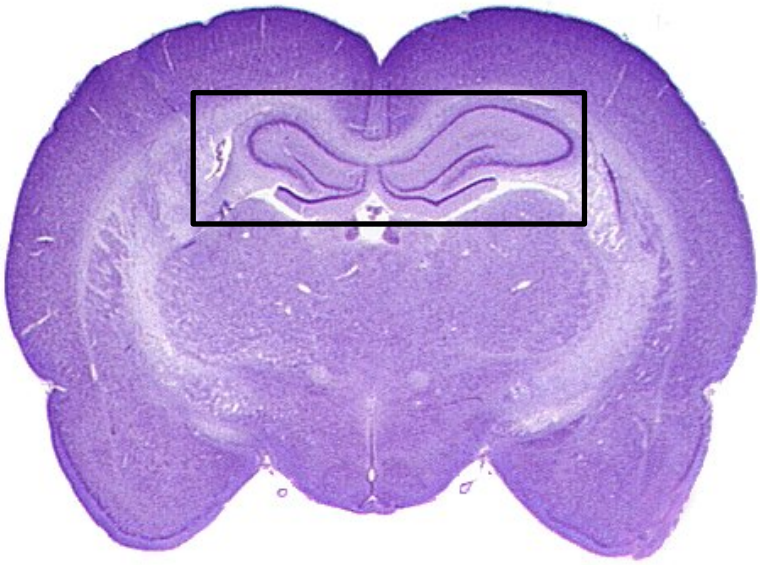

Figure 2*: Coronal section of the rat brain, including a highlight of the hippocampus. Adapted from SynapseWeb by John C. Fiala and Josef Spacek (15).*

The hippocampal region is unique, based on the fact that neurogenesis proceeds even in adulthood. "Adult hippocampal neurogenesis" is a term introduced by Goldman and Nottenbohm in 1983 (16), and reflects the process by which new functional neurons are generated from adult neural precursors in the dentate gyrus (17). As a result of aging, neurogenesis declines. This might contribute to neurodegenerative diseases, including AD (18). However, neurogenesis can also be enhanced as a compensatory reaction to neuronal damage representing an endogenous brain repair mechanism (19). The dentate gyrus is considered to contribute to the formation of new memories. Development of new neurons can be promoted by exercise.

Exercise seems to provide a noticeable increase of new neurons, furthermore, the favourable effects from exercise, such as cognition and mood, might be indirectly linked to specifically enhanced hippocampal neurogenesis (20).

It has become well-established that adult neurogenesis persists throughout life in restricted brain regions in mammals, more specifically in the subgranular zone in the dentate gyrus of the hippocampus, as illustrated in the figure below (figure 3) (21). However, adult hippocampal neurogenesis attracts the most attention, because it is considered to contribute to higher cognitive function, most notably the formation of new memories and emotional behaviour (22). Neurogenesis has been shown to be highly regulatable, the process can be modified by various factors, including age, stress and physical activity (20).

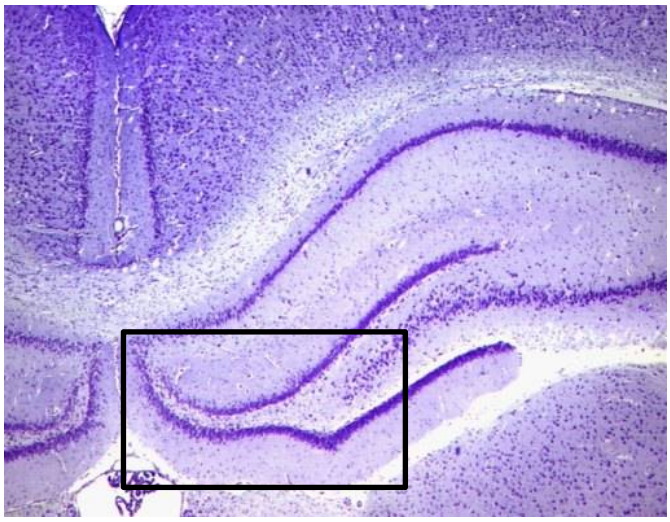

Figure 3*: Areas within the hippocampus, including a highlight of the dentate gyrus where adult neurogenesis mainly occurs. Adapted from SynapseWeb by John C. Fiala and Josef Spacek (15).*

The majority of research using a transgenic mouse model of AD (APPswe/PS1dE9) has repeatedly reported a reduction in adult hippocampal neurogenesis, giving rise to the idea that impaired neurogenesis is associated with further AD pathological changes (23). The pathological damage to the hippocampus encompasses the dentate gyrus, where adult neurogenesis occurs. This damage lead to an impaired neurogenesis (24), providing a decline in structural and functional plasticity in the hippocampus, which by definition is the brains ability to regulate its network structure as a response to the environment (25, 26).
In addition, the major constituent of senile plaques, Aβ, is considered to induce neuronal cell death.

This extensive loss of neurons is associated with hippocampal atrophy, creating a direct path to memory dysfunction (27). Following a certain pattern of regional spread, the neuropathologic changes gradually affect other regions of the brain (28).

1.2.1 Plasma factors rejuvenates the aging brain

Studies have shown that blood from young mice can reverse some effects of AD pathology in old mice. The hippocampus is particularly vulnerable to aging in both humans and mice, resulting in downregulation of plasticity-related genes, reduced spine density, decreased synaptic plasticity and impairments related to cognitive functions. Some soluble plasma factors are specifically associated with inhibiting and promoting brain neurogenesis. The administration of plasma from young rats has shown to improve hippocampal-dependent learning and memory, which might reduce the risk of a variety of neurodegenerative diseases. In contrast, exposure to blood from elderly mice has shown a significant reduction in adult hippocampal neurogenesis (13).

Furthermore, evidence has shown that physical exercise elevates the plasma levels of factors associated with cognitive function in confirmatory to blood from young rats (29). Exercise-induced factors such as BDNF, IGF-1 and vascular endothelial derived growth factor are the principal growth factors shown to enhance neurogenesis and to benefit in maintaining brain function and health (30). Taken together, circulatory soluble factors elevated in plasma after physical activity may have a therapeutic and disease-slowing effect for neurodegenerative diseases, such as AD (13).

1.3 Immunohistochemistry

IHC is an important and widely used method both in clinical diagnostics and in medical research laboratories. The IHC method uses specific antibodies targeting corresponding antigens present in the tissue, allowing for determination of the tissue distribution of a specific antigen of interest. This immunological method is widely used in many fields, such as in cancer diagnostics, genetics, for predicting therapeutic response, for confirming the presence of infectious agents, and in pathology, such as neuropathology (31).

Detection and visualization of the antigen-antibody complex can be achieved using a substance labelled to the antibody. This has to be done to identify if a reaction has occurred, and if so, where. There are different ways to detect this.

One of them is the immunofluorescent technique, where the complex is then labelled with a fluorescent marker (fluorophore) and will therefore be visible through a fluorescence microscope. One can also use immunoenzyme technique, where an enzyme evokes a colored complex, that is detectable in a regular light microscope (32).

1.3.1 Immunofluorescence

IHC is staining performed on tissue obtained by biopsy. The biopsy is fixated, processed and sectioned using a microtome. Further, the tissue sections are incubated with appropriate antibodies. When labelled with a fluorescent dye, the antigen-antibody binding can be visualized using a fluorescent microscope. This is called immunofluorescence (IF) (31). Both direct and indirect immunofluorescence staining are major techniques within IF. The direct method uses directly labelled primary antibodies. The indirect method entails an unlabeled primary and a labelled secondary antibody. The unlabeled primary antibody targets a specific epitope present in the tissue, and the labelled secondary antibody binds to the respective primary antibody. The direct method is time- and moneysaving, but since the marker is labelled on the primary antibody, the signal is not amplified. In contrast, the indirect method provides a signal amplification by binding multiple secondary antibodies to the primary antibody increasing the number of fluorophores per antigen. This makes the indirect method more sensitive and more effective than the direct method (33).

In this study project, the indirect immunofluorescence method was used. This method is used primarily on frozen tissue sections, which requires specialized equipment such as a freezing microtome. The figure below illustrates the schematic indirect immunofluorescence structure (figure 4).

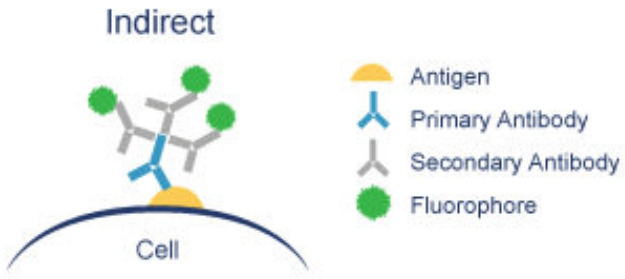

***Figure 4**: A schematic description of the indirect immunofluorescence staining technique. Adapted from Abcam (34).*

1.3.2 Fluorescence microscopy

The technique of fluorescence microscopy has become an essential tool to monitor properties of substances, organic or inorganic, such as detecting antigen-antibody complexes. Typical components of a fluorescence microscope are a light source, mainly mercury vapor or xenon arc lamps with an excitation filter, or lasers. The basic function of a fluorescence microscope is to irradiate the specimen with a high energy light of a specific band wavelengths which excites fluorophores, causing them to emit lower energy fluorescent light (35). A dichroic mirror acts as a wavelength specific filter, allowing light of specific wavelength to transmit trough to the detector, while light of other wavelengths is reflected back towards the source (36). A spectral emission filter separates the much weaker emitted light from the excitation light, which is seen by the eye or by another detector (35). A schematic description of the fluorescence microscopy is illustrated below (figure 5). A phenomenon known as autofluorescence occurs when cells are irradiated by visible light. This is a natural emission of light originated mainly from mitochondria and lysosomes, used to distinguish added fluorophores from cell autofluorescence (37).

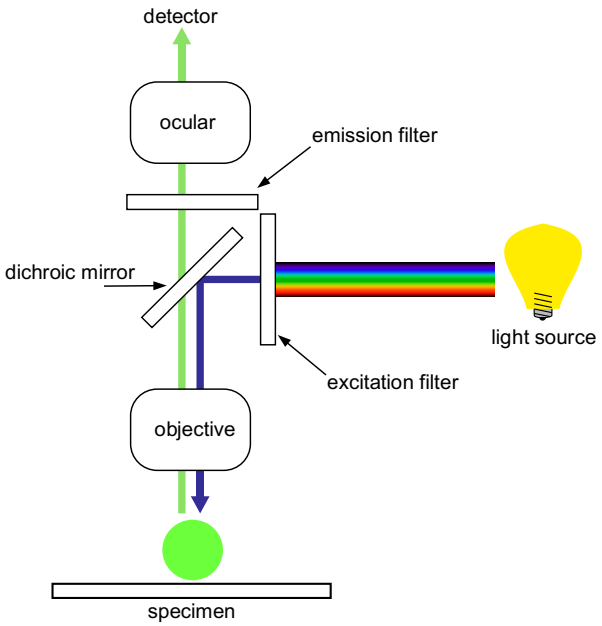

***Figure 5**: A schematic description of a fluorescence microscope (38).*

Fluorochromes have enabled cells and cellular components to be identified with a high degree of specificity among non-fluorescing material. Due to this, these components are ideal when detecting a structure of interest. Furthermore, the technique is capable of revealing the presence of a single molecule with a high resolution.

The disadvantage of fluorescent microscopy is that fluorophores lose their ability to fluorescence when illuminated due to photobleaching. Photobleaching is the irreversible destruction of the fluorophore that occurs as the molecules accumulate chemical damage from the electrons excited. Also, only the specific structure that has been fluorescently labelled can be visualized (35). Another limitation is that visualisation of intracellular structures requires that the cells have to be fixed as antibodies cannot penetrate through a cell membrane (39).

1.3.3 BrdU assay for detecting neurogenesis in an AD rat model brain

BrdU staining is a commonly used assay for studying adult neurogenesis. BrdU is an analogue of thymidine, which incorporates into the DNA of proliferating cells, during the S-phase of the cell-cycle. The thymidine analogue is detected using immunohistochemistry, as it integrates to single-stranded DNA, and therefore binds to a specific monoclonal antibody (40). BrdU will linger once it has been incorporated to the newly synthesized DNA, and as the cell divides, the thymidine analogue is passed down from generation to generation.

Most commonly, the research subject is injected with BrdU intraperitoneally. This specific step is also one of the drawbacks of this method, which includes stressful injections, in addition to an uncertainty if the targeted cells have been perforated or not. Stress significantly decreases proliferation of granule cell precursors in the dentate gyrus (1). Another disadvantage with this method is the fact that BrdU has undesirable side effects, as it is mutagenic and toxic. This substance changes the DNA-stability and might trigger cell death and lengthen the cell cycle, all with possible profound consequences for neurogenesis. Apart from this, the technique is to this date the most utilized when researching neurogenesis in the adult mammalian brain. This assay has provided researchers the opportunity to monitor proliferative neurons, in addition to their time of origin. BrdU assays have contributed to confirm the occurrence of adult neurogenesis, also in humans (40).

1.3.4 Detection of incorporated BrdU

When detecting newly synthesised neurons in the rat brain, it is important to make sure that the specific antibodies that are used detect the epitope present in the tissue, in addition to visualizing the antigen-antibody complex. Another important step is the incubation with serum, prepared from the same species as the host of the secondary antibody.

This includes a blocking reaction, added to prevent nonspecific background staining in the brain tissue, giving the assay higher specificity (41). Other critical steps include incubation time, temperature during incubation and concentration of the used antibodies (42). Performing an indirect immunofluorescence, the primary antibody should not be raised in the same species as the species of the sample to avoid cross-reactivity. It is therefore appropriate to use e.g. a mouse anti-BrdU primary monoclonal antibody if you are staining tissue from rat like in our case (43). A monoclonal antibody specifically binds to the same epitope on the antigen, which reflects in low cross-reactivity. The next step includes the use of a polyclonal secondary antibody, which binds to the primary antibody (anti-BrdU) already present in the tissue and bound to the corresponding antigen in the tissue (thymidine analogue) (44). A polyclonal antibody recognizes multiple epitopes on an antigen, which provides a high affinity against the primary antibody (43). The second antibody should be directed against the species of the primary antibody, and be conjugated with a fluorescent marker, for instance the fluorophore Alexa Fluor 488 (44).

Alexa Fluor 488 is a green-fluorescent dye with an excitation peak at λ-488 nm equivalent to blue, and an emission peak at λ-519 nm equivalent to green. BrdU-positive neurons are visualized on coronal sections of the brain using a fluorescence microscope detecting the peak wavelength of the green fluorescent at approximately λ-488/519nm (45). When contemplating the results, it is of high importance to compare the encountered fluorescence with both a positive and negative control, this to avoid false positive results and to confirm that any negative results are valid (46, 47).

1.3.5 Immunohistochemistry protocols

To assure that the specific antibodies that are used detect the epitope present in the tissue, in addition to visualizing the antigen- antibody complex, a detailed protocol corresponding to the particular lab, the specific AD rat model that is used, and the conditions at the laboratory are of great importance (48, 49). A protocol is a written document describing a method, such as an IHC assay. Detailed protocols are needed to minimize the analytical variation and to optimize the correctness and reproducibility. The protocol is a detailed description of a procedure, developed at the specific place where the assay is to be performed. The purpose of a protocol is to guarantee a valid result, no matter who performs the analysis, in other words, without inter-individual differences (50).

When developing a new staining method at a laboratory, a protocol has to be established. It is necessary to do adjustments from recommended protocols to optimize the procedure at the performing laboratory to ensure reproducible and successful results (51).

1.3.6 Immunohistochemical (IHC) controls

When performing an IHC assay, use of controls are essential to confirm that the observed staining result is valid. The positive control includes a section from a tissue expressing the protein or antigen of interest. The purpose of using a positive control is to verify that the observed staining is specific, and that the procedure is working and optimized. Optimally an IHC assay should also include a negative control to exclude nonspecific binding of the secondary antibody (52).

1.4 McGill-R-Thy1-APP

A variety of genetically modified animal models have been established and used in AD research. It has been verified that early onset familial AD is caused by single gene mutations, located in genes the AβPP and PSEN1/2 (5). Even though studying genetically modified animal models is not optimal in AD research, previous research findings have been of great advantage in the understanding of the underlying mechanisms of AD. Both mouse and rat models have been described, but as of today rat models are most often used. Rats imply to have several advantages compared to mice, most importantly they are considered genetically and physiologically more comparable to humans (53). AD is a complex disease and developing a transgenic animal model who suffers from both tau pathology and amyloid plaque at once has been difficult.

The McGill-R-Thy1-APP rat carries mutations in amyloid β-protein, derived from one of the isoforms of the amyloid precursor protein, AβPP751 (54). Mutations in this single transgene includes both the Indiana (V717F) mutation and the Swedish (KM670/671NL) double mutation. The murine Thy1.2 promoter controls both of these mutations and is regulated by the development of the brain. An adult rat brain will have higher levels of the murine Thy1.2 promoter, whereas a neonatal rat brain will have lower levels expressed (55). The McGill rat model displays intracellular amyloid-β accumulation, visible as early as postnatal day 7. At 6-9 months, the first amyloid β plaque deposit can be detected in the hippocampus (53). Additionally, this transgenic model also displays cognitive deterioration.

Previous studies show that even though there is little to no Aβ plaque present at 3 months, several cognitive functions are found altered at this early stage in life (56).

The study project uses the McGill-R-Thy1-ApP rat to characterize the early stages of cognitive decline and how the decline develops as the disease progresses. This is helpful to investigate whether blood from healthy and exercised trained rats has a positive impact on neurogenesis in rats with AD.

1.5 Topic statement

Since the exposure of blood products is considered as a novel potential AD prevention and therapeutic treatment because of its positive impact on neurogenesis, techniques on how to detect neurogenesis have to be optimized in every laboratory. The aim of this study is to establish an optimized staining method to detect newly synthesized neurons in the rat brain to be used at the laboratory at CERG. This leads to the topic statement:

"The establishment of an immunohistochemical staining method with anti-BrdU for detection of neurogenesis in an AD rat model brain."

2.0 Materials and methods

2.1 Quantitative method of research

We used an experimental quantitative research design. This included a systematic and scientific approach in which we measured a set of variables in order to establish and optimize the technique.

2.2 Chemical substances, equipment, reagents, antibodies

***Table 1**: An overview of the sample material, the McGill-R-Thy1-APP rats, used in the experiment*

Number	Identity	Injected treatment	Weight at tissue/brain collection (g)
2	9A1BB	Saline	320,0
3	B336F	Diluted plasma 1:1	286,9
5	A1426	Diluted plasma 1:1	238,3
6	9ECB3	Diluted plasma 1:1	321,4
7	B3956	Diluted plasma 1:1	302,8
10	C6650	Diluted plasma 1:1	300,8

All rats received twelve separate injections with BrdU in addition to the plasma treatment injections.

***Table 2**: Overview of chemical substances*

Chemical name	Formula	Supplier
Sodium dihydrogen phosphate	$NaH_2PO_4*H_2O$	Merck
Sodium hydrogen phosphate dihydrate	$Na_2HPO_4*2H_2O$	Merck
Tris(hydroxymethyl)-aminomethane	$C_4H_{11}NO_3$	Merck
Sodium chloride	NaCl	Merck
Hydrochloric acid fuming 37 %	HCl	Merck
Sodium Hydroxide	NaOH	Sigma-Aldrich
Triton X-100	$C_{14}H_{22}O(C_2H_4O)_n$	Sigma-Aldrich
Boric acid	H_3BO_3	Sigma-Aldrich
Vectashield H-1000 LOT: Z0806	-	Vector Laboratories

***Table 3**: List of laboratory equipment*

Equipment
6-well plate
Strainer
Cryotubes 1,5 mL
Paint brush
Petri dish
Olympus BX41 Microscope
Olympus U-RFL-T
Thermo Scientific Microtome HM430

***Table 1**: Reagents used for immunostaining*

Chemical name	Concentration	pH	Volume
DMSO buffer (2% DMSO, 20% glycerol, 0.1M Phosphate buffer)	-	-	100 mL
Phosphate-buffered saline (PBS)	0,4 M	7,4	500 mL
Phosphate-buffered saline (PBS)	0,125 M	7,4	500 mL
Tris-buffered saline (TBS)	-	8,0	500 mL
Sodium borate buffer	0,1 M	8,5	100 mL
TBS-Tx	0,5 %	8,0	500 mL
Tris-HCl	-	7,6	500 mL
Goat serum in TBS-Tx	10 %	-	10 mL
HCl	2 M	-	50 mL
NaOH	10 M	-	50 mL

See detailed description of solution preparation in Appendix I.

***Table 2**: Antibodies used for immunostaining*

Antibody	Recommended dilution from the supplier	Used dilution	Clonality	Supplier
Anti-BrdU antibody [IIB5] ab8152	1:5 - 1:10	1:10	Monoclonal	Abcam
Goat Anti-Mouse IgG H&L (Alexa Flour 488) ab150113	1:200	1:200	Polyclonal	Abcam

Anti-BrdU antibody [IIB5]:
Anti-BrdU is a monoclonal antibody produced in mouse. This primary antibody detects BrdU incorporated into DNA in reduplicating cells.

Goat Anti-Mouse IgG H&L (Alexa Flour 488):
Goat Anti-Mouse IgG H&L is a polyclonal antibody produced in goat, specific to mouse IgG. This secondary antibody binds to antibodies raised in mouse, such as Anti-BrdU antibody.

2.3 Preparatory work

In our project work, we used the McGill-R-Thy1-APP rat model provided by Professor Menno Witter at the Kavli Institute for Systems Neuroscience and Centre for Neural Computation at NTNU in Trondheim. In total six of the McGill-R-Thy1-APP rats were injected intraperitoneally with BrdU. In addition, the rats were injected with either Saline (control-group), or plasma from exercised or unexercised rats (control-group). The rats were euthanized at the age of 3 months, fixed in paraformaldehyde (PFA) using the intracardial perfusion technique, and the brains were extracted. At the time of tissue collection, the rats weighed between 238-322 g. The volume of BrdU injection was calculated based on the weight of the individual rat. Brains were stored separately in a Dimethyl sulfoxide (DMSO) buffer, before they were coronally sectioned.

2.4 Sectioning of brain tissue

The tissue sampling was performed at Kavli Institute for Systems Neuroscience at NTNU. The brains were sectioned coronally, using a freezing microtome, the Thermo Scientific Microtome HM430. Solid carbon dioxide (dry ice) was used to freeze the tissues, optimally to - 37 °C. The brains were sectioned in six series, meaning every sixth section was put in the same tube or mounted, each with a thickness of 40 µm. Each and every one of the series consisted of approximately 300 sections. In total six rat brains were sectioned, which resulted in about 2000 sections. Just below 20 of the sections were used during the protocol trials, meaning that remaining sections were stored in a DMSO buffer solution for future research. The first series was deposited into a petri dish filled with Tris(hydoxymethyl)aminomethane hydrochloride (Tris-HCl) before mounted on a Superfrost glass slide. The remaining five series of brain sections were put into tubes containing DMSO buffer to minimize tissue damage and stored for later studies. To orientate the brain sections, the "The Rat Brain in Stereotaxic Coordinates, 6th edition" atlas was used.

2.5 Immunostaining of newly synthesized neurons using BrdU assay

In total, six rat brains (fixated in PFA and sectioned) were stained in this study. The immunofluorescent staining was performed on free-floating sections using the anti-BrdU primary antibody [IIB5] ab8152, specific for BrdU incorporated into the DNA in the nucleated cell. Four sections were chosen from a cryotube containing all the coherent sections from the same rat. Since this was an animal study, every brain section is of high value. For this reason, we had to use sections from several rats during the optimization. If correctly done, every trial should be performed on the same rat, as it would minimize the number of uncertain variables when troubleshooting. In this selection process, all of the sections were put in a petri dish containing a DMSO buffer. Out of all these, four brain sections containing the hippocampus were chosen. The chosen sections were transferred to a strainer and rinsed in TBS. Subsequently, sections were incubated in preheated HCl (2 M) to denature DNA. To neutralize and remove the acid, the sections were rinsed in sodium borate buffer (0,1 M, pH 8,5). Next, the sections were rinsed in TBS to eliminate excess solution. To block non-specific binding from the secondary antibody made in goat, the sections were incubated in 10 % goat serum in TBS-Tx at room temperature prior to the primary antibody incubation. Sections were incubated with the primary antibody, Anti-BrdU, diluted 1:10 – 1:100 in TBS-Tx.

The following day, the sections were rinsed in TBS-Tx. Fluorescence antibodies are light sensitive and will fade when exposed to ambient light. For this reason, the next steps were performed in the dark to reduce the chances of photobleaching. The sections were incubated with the secondary antibody, Goat Anti-Mouse IgG H&L Alexa Flour 488, in a 1:200 dilution of TBS. The subsequent step was to rinse the sections in Tris-HCl to maintain a stable pH. Finally, the sections were floated in a petri dish in TBS before mounted on microscope slides in correct anatomical order. We used Vectashield as a mounting medium to prevent rapid photobleaching before sealing the coverslip with nail polish.

2.6 Detection of newly synthesized neurons

The glass slides with BrdU stained brain tissue were thoroughly studied using a fluorescence-microscope. The Olympus BX41 Microscope was used with the mercury burner power supply unit Olympus U-RFL-T. To examine the tissue sections, the objective lenses 10x/0,25, 20x/0,40 and 40x/0,65 were used. The excitation fluorescence filter was set to narrow-band blue-light, ideally suited to the wavelength 488 nm.

2.7 Troubleshooting process

Table 3: *Overview of completed troubleshooting, the adjustments performed during the multiple trials of the IHC staining method*

		First trial	Second trial	Third trial	Fourth trial
Anti-BrdU antibody	*Time (days)*	1	1	1	2
	Concentration	1:10	1:10	1:100	1:100
	Temperature (°C)	4	4	4	Room temperature
Goat Anti-Mouse IgG H&L Alexa Fluor 488	*Time (hours)*	1	1	2	2
	Concentration	1:200	1:200	1:200	1:200
	Temperature	Room temperature	Room temperature	Room temperature	Room temperature

3.0 Results and discussion

The main result of the study was an optimized BrdU Immunostaining technique designed to be used at the laboratory at CERG. The results can be divided into four trials, the results of each trial described and discussed in detail below.

3.1 First trial of the BrdU staining method

The first step in optimizing this method was to combine and adapt protocols from previous studies (appendix II and III) and Abcam´s IHC staining protocol. Tissue sections from rat number three (table 3) was used. The microscopy of the tissue sections showed poor staining, meaning no specific staining in the BrdU incorporated cells besides the autofluorescence (figure 6). The first trial of the procedure was concluded not successful.

Usually during IHC staining methods, an antigen retrieval step is necessary because fixation can lead to cross-links that masks the antigenic sites. The retrieval step reverses some of the cross-links and enables the antibodies to access the protein of interest within the tissue. Based on the fact that BrdU incorporates into replicating DNA in the nuclei of cells, the fixation does not affect the BrdU binding sites. Therefore, the unmasking prior to staining with primary antibody was not necessary. In contrast, the anti-BrdU assay requires treatment with heated HCl to denature DNA. Until this step is done, anti-BrdU is blocked, and does not have access to BrdU subunits, due to the base pairing in double stranded DNA. For this reason, the antigen retrieval step was replaced, and a denaturation step followed by an acid neutralizing step using sodium borate buffer was inserted.

This substitution allows the anti-BrdU antibody to access to the BrdU epitope incorporated within the DNA. In addition, the rinsing in PB was replaced with a rinsing in TBS, recommended by Abcam´s IHC staining protocol. The primary antibody concentration was not changed, because the dilution used, 1:10, was the recommended dilution from the supplier. The secondary antibody has previously shown successful results using the 1:200 dilution at the laboratory at CERG and was therefore trusted to be accurate in this assay.

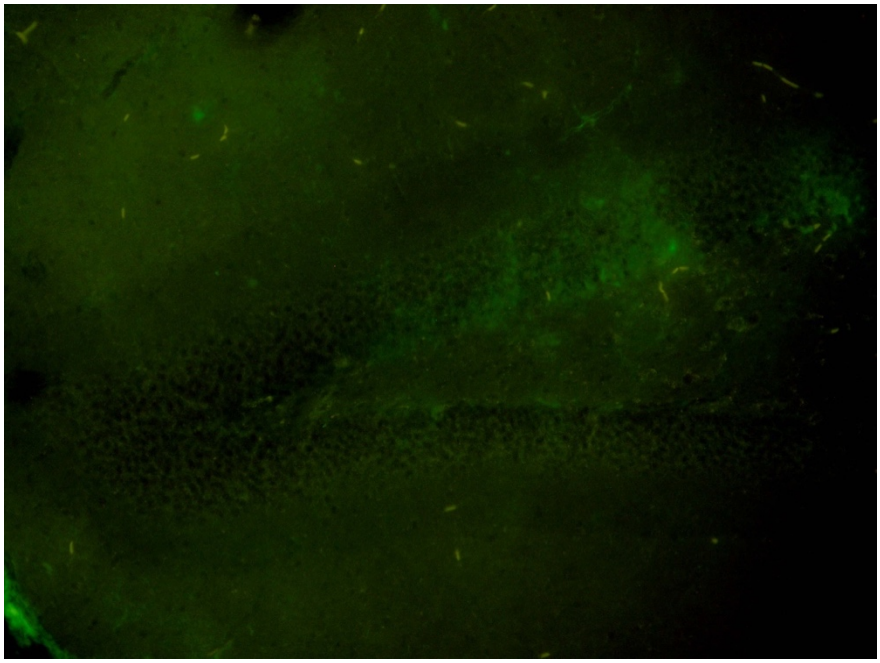

***Figure 1**: Negative BrdU staining of brain number three, dentate gyrus*

3.2 Second trial of the BrdU staining method

The adjustments for the second trial included replacing some of the steps in the first trial of the protocol. To carry out these adjustments, the brain of rat number two (table 3) was used. The second attempt also resulted in no specific staining in the BrdU incorporated cells (figure 7).

The greatest changes for the second trial were made in the initial phase of the protocol, whereas the unmasking of the tissues was substituted with an incubation in HCl to denature DNA. This substitution allows the antibody to access to the incorporated BrdU. Furthermore, the sections were rinsed in a sodium borate buffer and TBS. The following steps were identical to the first trial of the procedure. Further, it was decided to increase the incubation time, on both the primary- and secondary antibody because longer antibody incubation time seems to be helpful for the antibodies to penetrate through thicker sections, like these 40 µm thick sections.

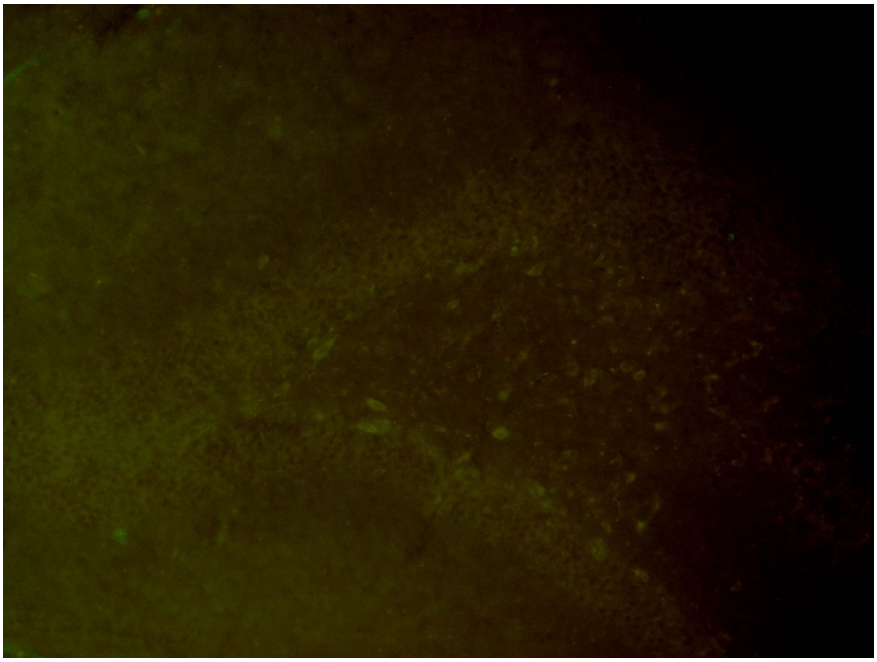

Figure 2: *Negative BrdU staining of brain number two, dentate gyrus*

3.3 Third trial of the BrdU staining method

In this attempt, two chosen sections from rat number five (table 3) were stained. The third attempt also resulted in no illuminating green cells as desired (figure 8).

At third attempt on optimizing this IHC method further adjustments were made, for example a decrease in the concentration of the primary antibody was addressed. This might seem illogical due to the fact that an increase in concentration promotes an antigen-antibody reaction, but prior studies have found that a decrease in concentration may lead to positive staining. Additionally, an increased incubation time was also added, as a common adjustment to promote a reaction.

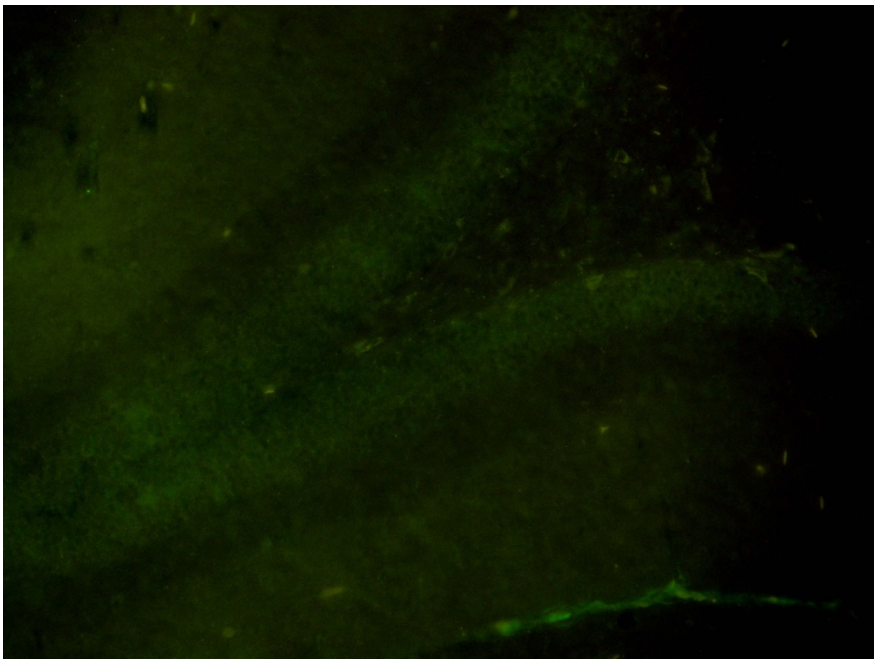

Figure 3: *Negative BrdU staining of brain number five, incubated for two days, dentate gyrus*

3.4 Fourth trial of the BrdU staining method

The final trial also resulted in no specific staining in the BrdU incorporated cells as well (figure 9).

The fourth attempt optimizing the BrdU assay was analogous to the third attempt, except the time of incubation. The primary antibody was incubated for two days as an attempt to increase the visualization of a possible antigen-antibody complex, by helping the antibody to detect the BrdU incorporated cells.

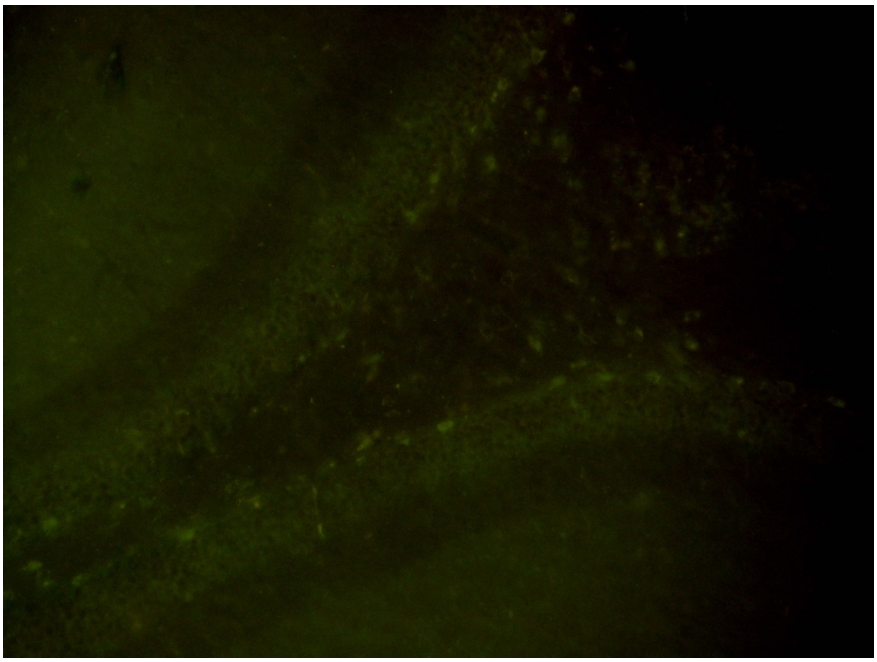

***Figure 4**: Negative BrdU staining of brain number five, incubated one day, dentate gyrus*

3.5 Control is key

First and foremost, performing an IHC staining without any control cannot be properly interpreted and complicates the troubleshooting process. In addition, an assessment of the observed staining pattern is impossible. Due to a delay caused by the supplier, the positive control did not show up in time. Therefore, the project had to be performed without the most essential part of the IHC staining protocol, namely the positive control. Considering the lack of a positive control, a verification of negative results is not valid. The possession of a positive control would ease the troubleshooting process. It would have provided an indication whether there is something wrong with the staining protocol itself, the tissue preparation or if the tissue is truly negative. Despite of this major drawback, the project was continued assuming that the source of error lies within the staining protocol, considering that the staining method contains a multiple of variables that can be changed in a limited timeframe. Optimally, a negative control should have also be included. Without one, one cannot conclude with certainty that the visualized fluorescence is due to interaction of the epitope of the antigen and the paratope of the antibody. Ideally, sections from a rat without BrdU injections should also serve as a negative control.

3.6 Hypothetical sources of error

3.6.1 Titration

Due to the lack of time and recourses, the troubleshooting to solve suboptimal IHC staining issues was inhibited. The most favorable choice would have been to determinate the optimal conditions for the staining by performing a titration of the primary and secondary antibody once arrived at the laboratory, as concentrations may vary across batches. This should be performed with the positive control. The titration process is helpful to determinate the correct concentration of antibody to use for this specific assay, as it helps ensure that the antibody gives the very best stain index.

3.6.2 Prior to IHC staining

The exact dosage of BrdU injection varies depending on the weight of the rat in use. Each rat received twelve separated injections of their assigned dosage. Repeated injections might be perceived as stressful experiences for the rat. Due to the fact that stress significantly decreases proliferation of granule cell precursors in the dentate gyrus, this is one of the major drawbacks of using BrdU.

The rats are injected multiple times which might cause a stress-induced decrease of hippocampal neurogenesis, hence resulting in poor or no positive staining. Another major drawback is the actual intraperitoneal injection, as there is no guarantee that the injection is correctly performed. Tissue fixation is also a critical step, as the antigens might become over-fixated leading to an unavailability of antigens and therefore lack in staining.

It is of great importance that the block temperature on the microtome is correct for the tissue being sectioned, to maintain high-quality tissue sections. As the tissue is frozen, it is important to avoid over-freezing, which is done by removing excess dry ice. This, however, is an economic disadvantage, as the dry ice will return to the gaseous stage upon storage, leading to regular deliveries and wasting of large amounts of dry ice. Also, a sharp edge knife is of great importance in obtaining a high-quality frozen section (57).

3.6.3 Time of incubation

During the four separate protocol testing and optimizing, varying incubation time with both primary- and secondary antibody were tested (table 6). An increase in incubation time may lead to a detection of the antigen-antibody complex, but this does not seem to be the case in this optimization study.

3.6.4 Antibody

One of the disadvantages of using IHC is the antibody itself, as it may be non-functional for the assay, e.g. the specificity of the antibody may not fit to the antigen of interest. In this case, the lack of a positive control makes this variable difficult to confirm. After several adjustments, and still no positive staining, the likelihood of the antibody being the source of error increases.

3.7 Final BrdU staining protocol for neurogenesis

Note: Carry out procedure with sections in a 6-well plate with strainers. All rinsing and incubations should be performed on a shaker in room temperature unless otherwise stated. Step 11 and beyond should be performed in the dark.

1. Carefully transfer brain sections to the strainer, using a paint brush
2. Rinse sections 3 x 5 min in TBS
3. Preheat HCl at 37 °C using a shaker
4. Denature DNA by incubating with 2 M HCl for 30 min at 37 °C
5. Rinse sections 10 min in 0,1 M sodium borate buffer
6. Rinse sections 3 x 10 min in TBS
7. Incubate sections with 10 % goat serum in TBS-Tx for 30 min
8. Draw off excess solution (do not wash)
9. Incubate sections in primary antibody, Anti-BrdU antibody, 1:10 in TBS-Tx overnight in a refrigerator at 4 °C
10. Rinse sections 3 x 10 min in TBS-Tx. Carefully extract the sections from the strainer using a paint brush
11. Incubate sections in secondary antibody, Goat Anti-Mouse IgG H&L Alexa Flour 488, 1:200 in TBS for 1 hour
12. Rinse sections 2 x 5 min in Tris-HCl
13. When ready to mount sections, float in TBS in a petri dish and arrange orientation of sections
14. Carefully transfer sections to slides using a paint brush
15. Add Vectashield and place coverslips
16. Seal coverslip with nail polish
17. Mounted tissues can be stored for up to 6 months in a refrigerator at 4 °C

4.0 Conclusion

During these past few weeks at CERG, we have performed a large number of tests for implementing a BrdU staining protocol for detecting neurogenesis in rats at their laboratory. We have tested a number of variables to optimize this protocol for rat brain tissue. This work has given a solid foundation to further trials for optimizing the staining protocol at the laboratory. First and foremost, the staining method must be performed with both a positive and negative control to confirm the achieved results. A complaint was sent to Abcam on the basis of assuming that the primary antibody did not fulfil the expected terms, but this is not known by certainty. A new primary antibody was sent from Abcam to be tested; however, it did not arrive in time during our project work. It might also be an idea to order the primary antibody from another supplier, since the suppliers differ in sensitivity and quality. When the BrdU staining protocol is fully optimized and established with a positive control to ensure valid results, further immunostaining can be performed on the brain tissue that has been sectioned and set aside during this project study.

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6.0 Appendixes

Appendix I: Solutions preparation for staining with BrdU

DMSO buffer

100 mL: 31,25 mL 400 mM phosphate buffer, 46,75 mL H_2O, 20 mL glycerine, 2 mL DMSO

Store in refrigerator for an extended period of time.

Phosphate buffer (PB) 0,4 M pH 7,4

A (acid): 13,8 g Sodium dihydrogen phosphate ($NaH_2PO_4*H_2O$)/250 mL H_2O

B (base): 35,6 g Sodium hydrogen phosphate dihydrate ($Na_2H_2PO_4*2H_2O$)/500 mL H_2O

Make solutions A and B. Add solution A to solution B until the pH is 7,4.

Store in the dark in room temperature for up to one month.

Phosphate buffer 0,125 M pH 7,4

Dilute 0,4 M phosphate buffer. 500 mL: 146 mL 0,4 M PB + 344 mL H_2O

Store at 4 °C for up to one week.

Tris-buffered saline (TBS) pH 8,0

500 mL: 3,03 g Tris + 4,48 g NaCl/500 mL H_2O

Adjust pH to 8,0 with HCl (2,0 M).

Store in refrigerator for up to one week.

TBS-Tx (0,5 %) pH 8,0

500 mL: 3,03 g Tris + 4,48 g NaCl/500 mL H_2O

Adjust pH to 8,0 with HCl (2,0 M).

In a ventilated hood, add 2,5 mL Triton X-100 and mix well.

Store in refrigerator for up to one week.

10 % goat serum in TBS-Tx

10 mL: 1 mL goat serum + 9,0 mL TBS-Tx

Dilute only prior to immediate use. Store aliquots of Goat Serum at -20 °C or below for an extended period of time.

Tris-HCl pH 7,6

500 mL: 3,03 g Tris/500 mL H_2O

Adjust pH to 7,6 with HCl (2,0 M).

Store in refrigerator for up to one week.

HCl 2 M

50 mL 8,33 mL 37 % HCl/41,67 mL H_2O

Do not make the mistake of adding water to concentrated HCl. Store in room temperature in a well-ventilated place for an extended period of time.

NaOH 10 M

50 mL: 20 g NaOH/50 mL

Should be stored in plastic containers. Store in room temperature in a well-ventilated place for an extended period of time.

Sodium borate buffer 0,1 M pH 8,5

100 mL: 0,62 g Boric acid/100 mL

Start from boric acid and dissolve it in 75 mL of H_2O. Adjust with concentrated NaOH (10 M and 1 M) until the pH is 8,5, and then top up to 100 mL with H_2O.

Store in room temperature for an extended period of time.

Appendix II: BrdU assay for neurogenesis in rodents

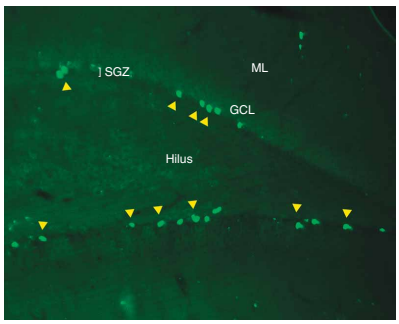


Figure 1 | BrdU-positive cells in a coronal section (rat, male, Sprague–Dawley, 35–40 d old, 10× objective) of the dentate gyrus. BrdU was injected intraperitoneally three times at 4-h intervals at 33.3 mg kg^{-1}. The brain was perfused 1 week following the last injection. Yellow arrows point to labeled cells in the subgranular zone (SGZ). GCL, granule cell layer; ML, molecular layer.

- Rat anti-BrdU primary monoclonal antibody (clone BU1/75, ICR1; Accurate Chemical & Scientific, cat. no. OBT0030); optimal dilution ratio 1:200
- Rabbit anti-human Ki67 polyclonal primary antibody (Vector Laboratories, cat. no. VP-K451); optimal dilution 1:200
- Mouse anti-human Ki67 monoclonal primary antibody (clone MIB-1; DakoCytomation, cat. no. M7240); optimal dilution 1:100
- Goat anti-DCX polyclonal primary antibody (C-18; Santa Cruz Biotechnology, cat. no. SC-8066); optimal dilution ratio 1:200
- Alexa Fluor 488 chicken anti-Rat IgG (H+L) secondary antibody (Molecular Probes); optimal dilution ratio 1:200
- Alexa Fluor 568 goat anti-rabbit/mouse IgG (H+L) secondary antibody (Molecular Probes); optimal dilution ratio 1:200
- Alexa Fluor 594 donkey anti-Goat IgG (H+L) (Molecular Probes); optimal dilution ratio 1:200

EQUIPMENT

- Dissecting tools
- Syringes
- 23–27G 1-inch needle, preferably with a short bevel
- Perfusion pump or 50-ml syringe
- Glass vials or 15-ml polypropylene conical tubes, 17 × 120 mm (Becton Dickinson)
- Vibratome/Microtome/Cryostat
- 24- or 6-multiwell tissue cell culture polystyrene plates
- Oven (37–45 °C)
- Shaker
- Microscope slides (VWR, cat. no. 48323-185; 76 × 26 mm)
- Micro cover-glasses (VWR, cat. no. 48404 454; 24 × 60 mm)
- Fluorescent microscope
- Confocal microscope

REAGENT SETUP

BrdU injection solution Warm saline solution (0.9% w/v NaCl in sterile H_2O) to 40–50 °C, slowly dissolve BrdU in saline solution by gently vortexing, allow the BrdU injection solution to cool to room temperature (22–25 °C) and use the BrdU injection solution immediately.

Storage at 4 °C is not recommended due to the formation of white BrdU precipitates (crystals). A 20 mg ml^{-1} stock is good for most cases that require intraperitoneal injections. ▲ CRITICAL White BrdU precipitates can be re-dissolved at 40–50 °C for later use. The solubility of BrdU in normal saline is pH-dependent and BrdU dissolved in saline is acidic; therefore, the addition of NaOH (0.01 M) may be required to keep the pH as neutral as possible. However, a basic solution may cause an adverse reaction with the animals' skin and tissue; therefore, heating and slowly dissolving BrdU while titrating the pH towards 7 without making the solution basic is preferred.

! CAUTION There is some evidence in the literature that BrdU may be carcinogenic and produce developmental abnormalities when given to prenatal and neonatal animals[27,28]. The compound should be handled in the fume hood. The solution of BrdU should be handled with gloves.

PBS, 0.1 M, pH 7.4 In a 1–2 L beaker, add 2.7 g of sodium phosphate monobasic NaH_2PO_4, 11.5 g of sodium phosphate dibasic Na_2HPO_4 and 9 g NaCl. Add distilled water up to 1000 ml and stir. Measure the pH, which should be around 7.4.

Blocking solution, 0.1 M PBS, 0.3% Triton X-100, 2% serum In a 1–2 L beaker, add 20 ml of serum, 3 ml of Triton X-100, 0.1 M PBS up to 1,000 ml and stir. Store in 50-ml aliquots at –20 °C. (Ideally, the serum should be taken from the species of animal in which the secondary antibodies are made. Alternatively, normal horse serum can be used instead of goat serum.)

Sodium citrate buffer, 10 mM, 0.05 Triton X-100, pH 6 In a 1–2 L beaker, add 2.94 g of tri-sodium citrate (dihydrate) and distilled water up to 1,000 ml and stir. Adjust pH to 6.0 with 1 M HCl. Add 0.5 ml of Triton X-100. Store at 4 °C for up to 6 months.

4% Paraformaldehyde in 0.1 M PBS In a 1–2 L beaker, add about 800 ml of 0.1 M PBS. Heat 0.1 M PBS to 60–65 °C while stirring. At 60–65 °C, add 40 g of paraformaldehyde powder slowly while stirring (note: adding a few drops of 1 M NaOH helps to keep the solution clear). Continue to stir until the paraformaldehyde powder is dissolved, making sure that the temperature stays between 60 and 65 °C. Cool the solution until it reaches room temperature. Filter solution and keep at 4 °C. ! CAUTION Prepare in the fumehood and keep in the fridge until use. Can be kept for several weeks.

PROCEDURE

BrdU injection

1| Inject BrdU solution intraperitoneally.

▲ CRITICAL STEP The ideal BrdU injection dosage varies from 50 mg kg^{-1} to 300 mg kg^{-1} depending on the experiment and the animal in use. Multiple 50 mg kg^{-1} injections are often used to ensure the labeling of many cells undergoing cell divisions over a period of time, usually several days (usually in mice). This procedure is adequate when the precise dating of cells is not required. Higher doses in the order of 300 mg kg^{-1} are preferred when the time-line of the experiment is short and the age of the cells is of essence. The higher dose also ensures that the BrdU is not 'diluted' in the process of re-division of daughter cells[1,16,22]. Using the stock solution described above, approximately 5 ml of BrdU solution is required for a 500 g adult rat to obtain 200 mg kg^{-1}.

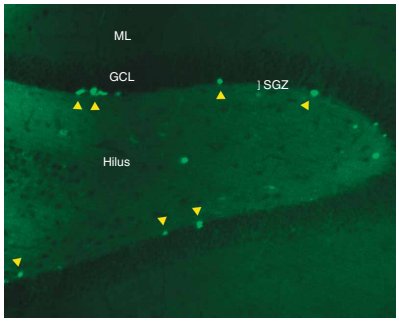


Figure 2 | Ki67-positive cells in a coronal section (rat, male, Sprague–Dawley, 35–40 d old, 10× objective) of the dentate gyrus. Yellow arrows point to labeled cells in the subgranular zone (SGZ). GCL, granule cell layer; ML, molecular layer.

■ **PAUSE POINT** For an intraperitoneal injection, the lower abdominal cavity must be isolated. In mice, we use a 27-gauge needle and a 1-ml syringe. The maximum intraperitoneal injection volume in the mouse is 3 ml. For rats, we use a 23-gauge needle and a 5-ml syringe. The maximum tolerable intraperitoneal injection volume in the rat is 10 ml.

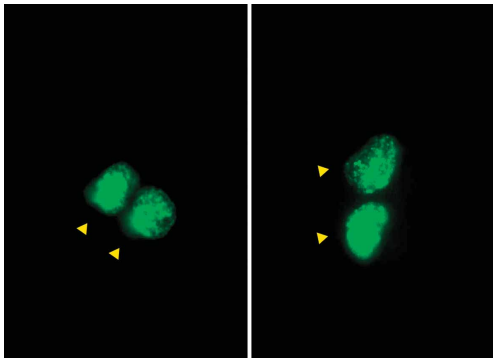


Figure 3 | Examples of BrdU (left) and Ki67 (right) positive nuclei shown at high magnification (100×). Images were taken 24 h after BrdU injection. (Obtained from Kee *et al.*, 2002 with permission from Elsevier.)

Anesthesia

2| Animals are first anesthetized with isoflurane. Apply approximately 1 ml of isoflurane to tissue paper and place it in a closed chamber. Immediately place the animals in the chamber and close the lid and wait for 1 min.

▲ **CRITICAL STEP** Isoflurane is a health hazard, so it should be handled with care. Proper protection, such as a lab coat, mask and goggles should be worn at all times. Moreover, as isoflurane is an inhalation anesthetic, all work involving isoflurane should be conducted under a properly ventilated fume hood.

3| After 1 min, monitor and test the animal to determine whether the animal is fully anesthetized and ready for transcardial perfusion. Fully anesthetized animals should display the following properties: respiratory rate (breathing) should be regular and relaxed; withdrawal reflexes should be absent (this can be tested by pinching one of the paws); and there should be no response to external stimuli (e.g., blowing air on the eye). If the animal still displays signs of awareness, place the animal back into the chamber for 1 more minute and repeat the monitoring and testing.

Transcardial perfusion

4| Expose the heart using sharp dissecting tools.

5| Insert the needle connected to the pump into the left ventricle. Make an incision in the right atrium to allow blood to flow out of the animal's body.

6| Perfuse the animal with PBS, 0.1 M at pH 7.4.

7| When the draining blood becomes clear, perfuse the animal with 4% paraformaldehyde in 0.1 M PBS.

▲ **CRITICAL STEP** The amount of 4% paraformaldehyde in 0.1 M PBS required for perfusion varies with the weight of the animal. Typically, a rat weighing 500 g will require 200 ml solution.

Dissection

8| Using appropriate dissecting tools, remove the head, then remove the muscle and membranous tissue from the top part of the skull and gently extract the brain from the skull.

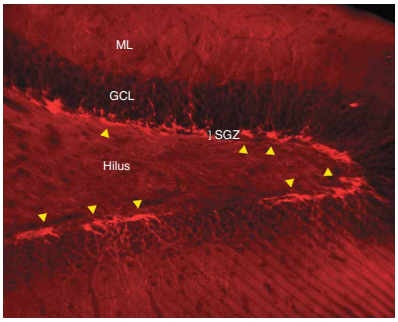


Figure 4 | Doublecortin-positive cells in a coronal section (rat, male, Sprague–Dawley, 35–40 d old, 10× objective). Yellow arrows point to labeled cells found in the inner granule cell layer (GCL). ML, molecular layer; SGZ, subgranular zone.

9| Cut the trigeminal and optic nerves, and let the brain fall into a beaker of cold 4% paraformaldehyde in 0.1 M PBS.

Post-fixation

10| Immerse the removed brain in the 4% paraformaldehyde in 0.1 M PBS for 24–48 h.

■ **PAUSE POINT** Tissues can be left for 1–2 d in the 4% paraformaldehyde in 0.1 M PBS at 4 °C.

▲ **CRITICAL STEP** Over-fixation may result in a lack of staining due to the unavailability of antigens.

Sectioning

11| Use the vibratome to section tissue into 10–40 μm slices.

▲ **CRITICAL STEP** Vibratome sections have some advantages when performing immunohistochemistry as the tissue is not processed further and keeps the antigenicity better than some other methods (e.g., paraffin embedding, ethanol fixing). Vibratome sections allow the morphology of tissue sections to be

un-disrupted due to no freezing and thawing steps. The disadvantage of vibratome sections is the slow and difficult sectioning step. In poorly fixed tissues, the formation of vibration marks or vibratome lines, which are often visible, may hinder analysis in the sections.

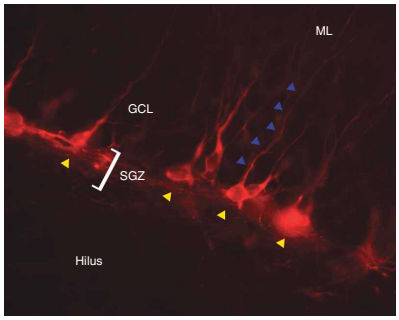


Figure 5 | Doublecortin-positive cells. As in **Figure 4**, but at higher magnification (40× objective) to illustrate the morphology of the immature neurons. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

DNA denaturation (required only for BrdU immunohistochemistry; otherwise, go to Step 16)

12| Transfer sections to 24-well plates loaded with 0.1 M PBS (at pH 7.4).

13| Rinse sections three times, 5 min each with 0.1 M PBS (at pH 7.4) on a shaker.

14| Denature DNA by incubating sections in 1 M HCl for 30 min at 45 °C (or 2 M HCl for 15 min at 37 °C).
▲ **CRITICAL STEP** Denaturation of DNA allows access for the anti-BrdU antibody so incomplete denaturation causes problems. There are various denaturation procedures (such as ethanol treatment and enzyme treatment), which may have different effects on the retention of morphology. The acid treatment with an increased temperature generally results in more effective exposure of the halogenated-nucleotide antigen. However, harsh HCl treatment (>2 M HCl), in conjunction with high temperature (> 65 °C) incubation, is detrimental to other antigens, particularly surface antigens and receptors. Moreover, some BrdU antibodies can recognize methylated thymidine under harsh denaturation conditions. This non-specific staining is evident when all or most of the nuclei are stained. Thus, careful adjustments of the denaturation conditions are necessary.
■ **PAUSE POINT** Earlier protocols have used a pre-treatment step using incubation with formamide at 65 °C, which precedes the HCl step; however, with the advent of new BrdU antibodies, this step is not required (see **Figs. 1,3**).

15| Neutralize the acid by rinsing sections three times, 5 min each with 0.1 M PBS (at pH 7.4) on a shaker.

Antigen retrieval (required for use with monoclonal Ki67 primary antibodies; otherwise, go to Step 21)

16| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4).

17| Transfer the sections to 10 mM sodium citrate buffer (at pH 6), preheated to 80 °C in a water bath.

18| Keep sections in 10 mM sodium citrate buffer (at pH 6) at 80 °C for 30 min.

19| Keep sections in 10 mM sodium citrate buffer (at pH 6) while allowing the sections to cool to room temperature.

20| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4).

BrdU, Ki67, DCX single immunohistochemistry

21| Transfer sections to 24-well plates loaded with 0.1 M PBS (at pH 7.4).

22| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4) on a shaker.

23| Incubate sections with blocking solution for 60 min at room temperature on a shaker.

24| Incubate sections with BrdU or Ki67 and/or DCX primary antibody diluted in blocking solution for 24–48 h at 4 °C on a shaker.
■ **PAUSE POINT** Tissues can be left overnight at 4 °C or longer.

25| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4) on a shaker.

26| Incubate sections with fluorochrome-conjugated secondary antibody in the dark, diluted in 0.1 M PBS (at pH 7.4) with 0.3% Triton X solution (detergent such as Triton-X can be added to the secondary antibody diluent to reduce hydrophobic interactions between tissue and reagent proteins, thus reducing non-specific binding of secondary antibodies) for 2 h at room temperature on a shaker.

27| Rinse sections three times, 5 min each in 0.1 M PBS (at pH 7.4) on a shaker.

28| If you are performing a single immunohistochemistry analysis only, go to Step 31 (and see **Figs. 1–5**).

Second immunolabeling for BrdU/DCX double immunohistochemistry

29| After following instructions from Steps 12–27 using BrdU primary antibody, proceed to Step 30.

30| Repeat the instructions from Steps 12–27 with the second primary antibody against DCX; then continue to Steps 31–32.
▲ **CRITICAL STEP** Double or triple-color immunostaining is complicated. It is important to choose antibodies that are compatible with the fixation, embedding and that will not cross-react. Performing single-color staining of the BrdU and the other markers (e.g., DCX, NeuN and CaBP D28K) prior to and in parallel with the double-color staining can be helpful. Once the optimal conditions for BrdU and DCX have been determined (titration), we recommend sequential reactions (BrdU staining first and DCX staining thereafter) for double immunostaining. The sequential reactions for double immunostaining have two main advantages over the simultaneous (addition of the BrdU and DCX antibodies together in a cocktail) double immunostaining reaction. First, the likelihood of cross-reaction between the secondary antibodies is reduced. For example, the order in which the secondary antibodies are added can be controlled to avoid non-specific binding (e.g., when using goat anti-mouse and donkey anti-goat secondary antibodies, donkey-anti-goat antibody reaction can be performed before the addition of goat anti-mouse to avoid non-specific binding). Second, each staining step can be verified before continuing onto the next step. Thus, for novices, this is a good way to troubleshoot in case of problems. In experienced hands, the sequential reactions can be performed simultaneously. In the latter case, a simultaneous double immunostaining reaction with properly chosen secondary antibodies (the secondary antibodies should ideally come from the same host to avoid cross-reaction) works well and saves time.

31| Carefully transfer sections to slides using a soft brush.

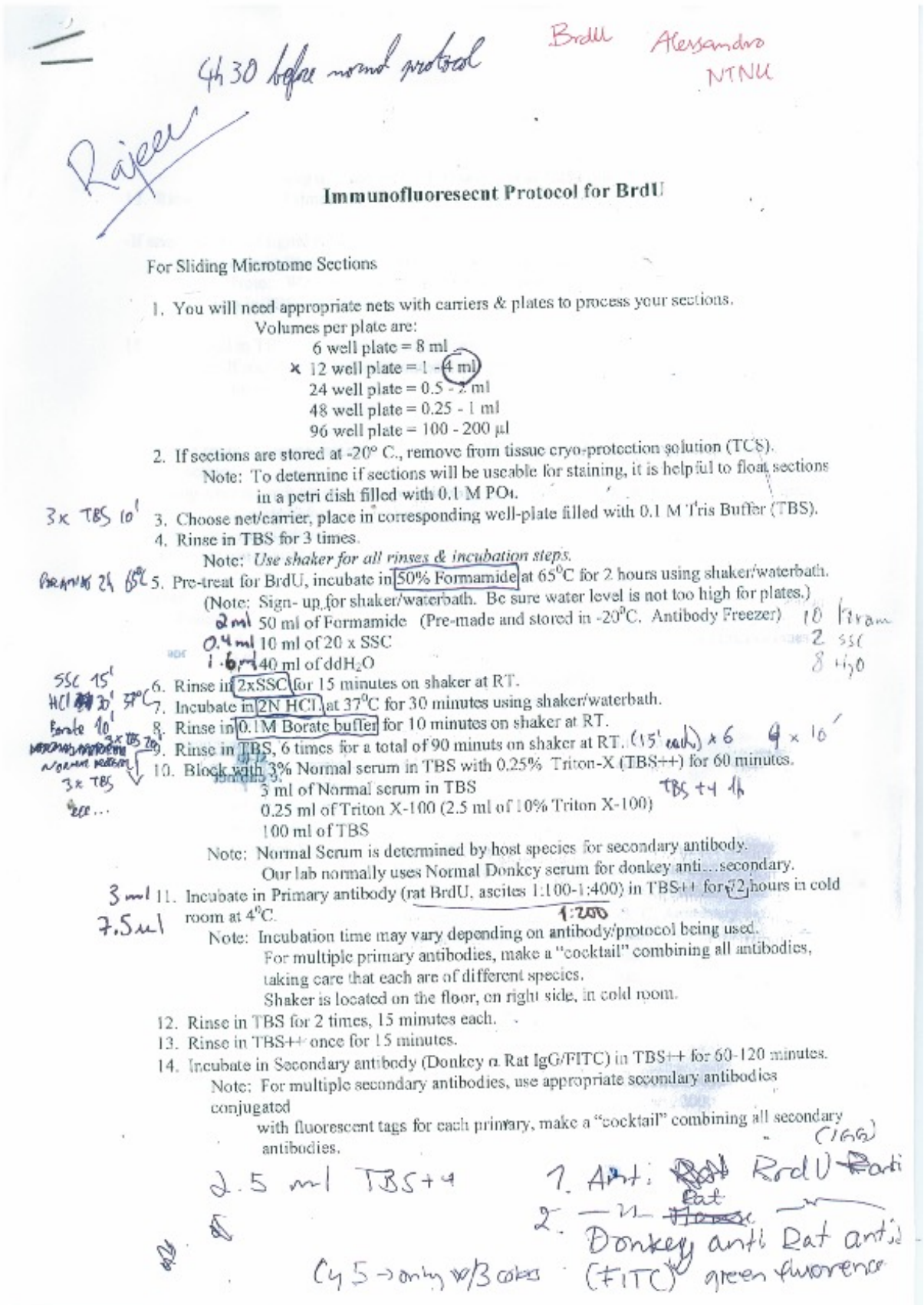
32| Add mounting medium (Permafluor) and place coverslips.
■ **PAUSE POINT** Mounted tissues can be kept at 4 °C for up to 6 months before imaging with a microscope.

? TROUBLESHOOTING
See **Table 1** for troubleshooting advice.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Action
BrdU/Ki67/DCX: Poor positive staining and/or no positive staining with little or no background staining.	BrdU/Ki67/DCX primary and secondary antibody concentration was not optimal.	Titrate BrdU/Ki67/DCX primary and secondary antibody when it arrives, as concentrations may vary across batches. This should be used with the tissue that serves as positive control.
	Incubation time with primary and/or secondary antibody was too short.	Use longer incubation times for primary antibody and/or secondary antibodies.
	The primary antibody does not recognize the antigen due to incorrect or over-fixation.	Include antigen retrieval step. Note: For BrdU primary antibody, over-fixation does not effect BrdU immunostaining.
	Antigen destroyed by excessive antigen retrieval step.	Reduce antigen retrieval time.
	Slides were left to dry.	Do not let slides dry out and keep wet at all times during the staining procedure.
BrdU: Poor positive staining and/or no positive staining with little or no background staining.	Denaturation of DNA by HCl was not sufficient.	Use higher concentration of HCl and/or longer incubation time. In addition, use increased temperature with HCl incubation.
Ki67: Poor positive staining and/or no positive staining with little or no background staining.	Antigen retrieval time was too short so that the Ki67 antigen was not available to Ki67 primary antibody.	Increase antigen retrieval time.
BrdU/Ki67/DCX: Non-specific and/or high background staining.	Non-specific binding of primary or secondary antibody.	Increase the number and time of washes in between steps.
	Incubation time with BrdU/Ki67/DCX primary antibody was too long.	Reduce BrdU/Ki67/DCX primary antibody incubation time.
	Blocking reaction was not optimal to allow for non-specific binding of the secondary antibodies.	Increase length and/or concentration of incubation with blocking solution made from the same species as the host of the secondary antibody.
	Aggregates binding.	Centrifuge antibody stock briefly in a micro-centrifuge at high speed to remove aggregates.

Appendix III: Immunofluorescent Protocol for BrdU



4h 30 before normal protocol

BrdU Alessandro NTNU

Rajeev

Immunofluoresecnt Protocol for BrdU

For Sliding Microtome Sections

1. You will need appropriate nets with carriers & plates to process your sections.
   Volumes per plate are:
   6 well plate = 8 ml
   x 12 well plate = 1 - 4 ml
   24 well plate = 0.5 - 2 ml
   48 well plate = 0.25 - 1 ml
   96 well plate = 100 - 200 µl
2. If sections are stored at -20° C., remove from tissue cryo-protection solution (TCS).
   Note: To determine if sections will be useable for staining, it is helpful to float sections in a petri dish filled with 0.1 M PO_4.
3. Choose net/carrier, place in corresponding well-plate filled with 0.1 M Tris Buffer (TBS).
4. Rinse in TBS for 3 times.
   Note: *Use shaker for all rinses & incubation steps.*
5. Pre-treat for BrdU, incubate in 50% Formamide at 65°C for 2 hours using shaker/waterbath.
   (Note: Sign- up for shaker/waterbath. Be sure water level is not too high for plates.)
   2 ml 50 ml of Formamide (Pre-made and stored in -20°C. Antibody Freezer)
   0.4 ml 10 ml of 20 x SSC
   1.6 ml 40 ml of ddH_2O
6. Rinse in 2xSSC for 15 minutes on shaker at RT.
7. Incubate in 2N HCl at 37°C for 30 minutes using shaker/waterbath.
8. Rinse in 0.1M Borate buffer for 10 minutes on shaker at RT.
9. Rinse in TBS, 6 times for a total of 90 minuts on shaker at RT. (15' each) x 6
10. Block with 3% Normal serum in TBS with 0.25% Triton-X (TBS++) for 60 minutes.
    3 ml of Normal serum in TBS
    0.25 ml of Triton X-100 (2.5 ml of 10% Triton X-100)
    100 ml of TBS
    Note: Normal Serum is determined by host species for secondary antibody.
    Our lab normally uses Normal Donkey serum for donkey anti...secondary.
11. Incubate in Primary antibody (rat BrdU, ascites 1:100-1:400) in TBS++ for 72 hours in cold room at 4°C.
    3 ml 7.5 µl 1:200
    Note: Incubation time may vary depending on antibody/protocol being used.
    For multiple primary antibodies, make a "cocktail" combining all antibodies, taking care that each are of different species.
    Shaker is located on the floor, on right side, in cold room.
12. Rinse in TBS for 2 times, 15 minutes each.
13. Rinse in TBS++ once for 15 minutes.
14. Incubate in Secondary antibody (Donkey α Rat IgG/FITC) in TBS++ for 60-120 minutes.
    Note: For multiple secondary antibodies, use appropriate secondary antibodies conjugated
    with fluorescent tags for each primary, make a "cocktail" combining all secondary antibodies.

2.5 ml TBS++

1. Ant: Rat BrdU Rat anti (IgG)
2. — " — Donkey anti Rat anti (FITC) green fluorence

Cy5 → only w/3 colors

All Donkey α... secondary antibody, use at 1:250 dilution.

15. Rinse in TBS for 3 times. x10 min 4 x 5' 1 x 10'

-If amplification of signal is needed:

a. Incubate in Streptavidin in TBS for 60-120 minutes.
   Note: We use it conjugated with fluorescent &diluted at 1:250.
b. Rinse in TBS for 3 times.

16. Rinse well in TBS for a minimum of 4 times.
   Note: If sections cannot be mounted, they may be stored in the cold room until ready to mount.

MOUNTING

17. When ready to mount sections, float in 0.1 M PO4 in petri dish & arrange orientation of sections.
18. Using a brush, pick-up sections on a double-subbed slide, making sure sections have adhered to slide before coverslipping.
19. Coverslip using Dabco/PVA.
20.. Dry overnite in cold room.

Supplies:	Source:	Storage:
Plates		
6-well	Stockroom #LS0640	On shelf above microtomes
12-well	Fisher # 07-200-81	
24-well	Stockroom #LS0630	
48-well	Stockroom #LS0635	
96-well	Stockroom #LS0650	
Net/Carriers	From Costar	In Cabinet next to Antibody file cabinet
15 mm diameter	#3481	
12-well carrier	#3520	
24 mm diameter	#3484	
6-well carrier	#3521	
Normal Donkey Serum	Sigma #D9663	-20° C. Antibody freezer
Triton X-100	Sigma #X-100	Chemical Shelf
Secondary Antibody	Jackson	-20° C. Antibody freezer
Streptavidin	Jackson	-20° C. Antibody freezer
Propydium Iodide	Molecular Probes #P-3566	Histo Chemical Refrig.
Dapi		Histo Chemical Freezer
Dapco/PVA		Histo Chemical Freezer

Linda Kitabayashi
January, 2000

PVA