2. Analysis of the structure and function of the mammalian retina; diseases of the eye

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2.1General Background:

Being able to see is very important for daily activities such as reading, watching television, playing sports and communicating with friends. Losing our sight, therefore, has a major impact on our quality of life. The eye allows us to see and interpret shapes, colour and dimensions but the eye does not actually see objects, instead it works similar to a camera. It absorbs light that objects reflect or give off, making it possible to see in bright light and in dim light, but not during darkness. An important part of the eye is the retina (from Latin rēte, meaning "net"); the light-sensitive tissue lining the inner surface of the eye, like the film of a camera. Light that enters the eye is changed into electrical signals via the retina. These signals are sent to the brain, via the optic nerve, which transforms them into visual images, like developing a film.

The retina and the optic nerve originate as outgrowths of the developing brain, so the retina is actually part of our central nervous system. As such, the retina is a structure with several layers of neurons interconnected by synapses. The neurons that are sensitive to light are called photoreceptors, of which there are two types: the rods and cones. Rods provide black and white vision and function in dim light, while cones provide colour vision during daytime. A human eye has approximately 125 million rods and 6.5 million cones, with a high concentration in a specialised area of the retina, called the macula. The macula is situated roughly at the centre of the retina. It is the focus for incoming light and is responsible for central vision and the ability to see detail.

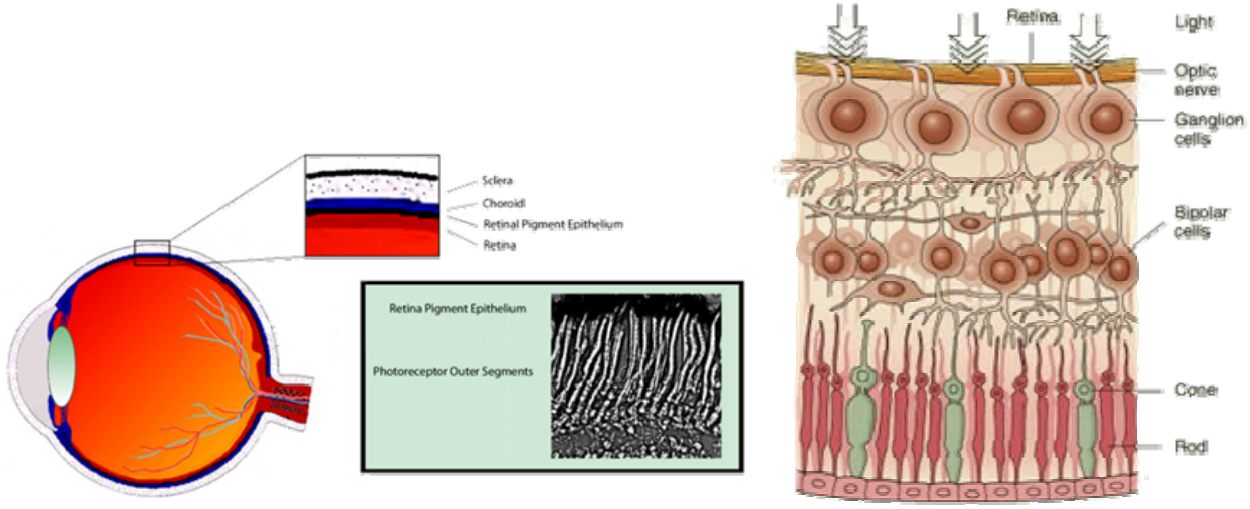

Figure 1. Photoreceptors form an important part of the retina, the light sensitive layers of the eye located in the back of the retina. Two types of photoreceptors exist: rods and cones, which allow us to see black, white and colour. From [*http://www.photobiology.info/Parker-Crouch.htm*](http://www.photobiology.info/Parker-Crouch.htm)

Anatomy of vertebrate retina

After light has been sensed by the photoreceptor, a chemical change in the rods and cones induces a signal that goes first to the bipolar and horizontal cells , then to the amacrine cells and ganglion cells and finally to the optic nerve fibres, where the signal enters the brain (see figure 2). The retina contains not only photoreceptors and ganglions cells, there are many 'support' cells, including epithelial cells, blood vessels and immune cells (the microglia). Together they form a complex network build in ten different layers. The retinal epithelial cells (RPE) form the most outer layer. This cell layer is very important for keeping the eye healthy: they provide the photoreceptors with oxygen from blood vessels that are located behind the eye, called the choroid, and remove debris from the retina that can cause damage. For example, photoreceptors are build out of discs, and each day new discs are formed and old discs are shed. The RPE cells make sure that the old discs are removed from the eye. Changes in the retina can lead to dysfunctional photoreceptors, leading to loss of vision. There are various risk factors associated with blindless, including age, inflammation, smoking and diet.

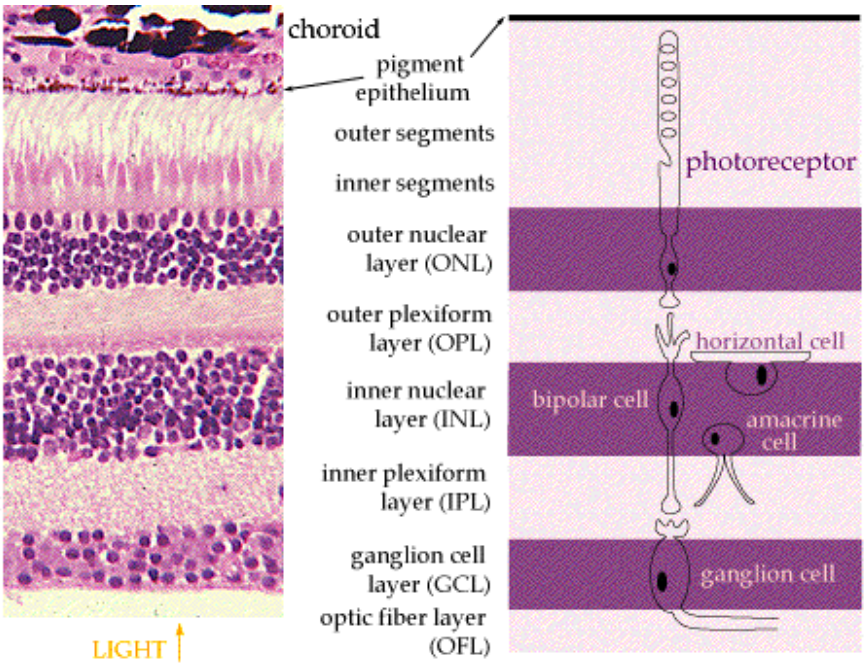

Figure 2: The layers of the retina.

Interesting differences have been reported for different animals. In birds, the pecten is a vascular structure of complex shape that projects from the retina into the vitreous humor; it supplies oxygen and nutrients to the eye, and may also aid in vision. Reptiles have a similar, but much simpler, structure, referred to as the papillary cone.

Statistics about sight loss and eye disease

The number of people living with sight loss is estimated to be 285 million, of whom 39 million are blind and about 90% of the world's visually impaired live in developing countries. It is believed that every 5 seconds one person in the world goes blind. In the UK, there are1.8 million people living with sight loss and about a third of people over 65 have difficulties with their eyesight. Every day around 100 people in the UK start to lose their sight.

Common eye diseases

Diabetic retinopathy

Diabetes occurs as a result of a lack of insulin or where the body is unable to process insulin properly. Insulin acts as a regulator of blood sugar levels. Diabetes induces damage to the blood vessels in the eye,

causing blindness. The blood vessels become weak, start leaking blood, leading to reduced oxygen delivery and damage to the photoreceptors. To compensate for the lack of oxygen, new blood vessels are generated, but these are also weak causing further damage and scar formation, which eventually leads to detachment of the retina and glaucoma (high pressure in the eye).

Age-related macular degeneration

Macular degeneration describes a condition where the macula does not function properly, and this disease mainly affects elderly, hence its name: age-related macular degeneration (AMD). AMD can occur in two different ways: "wet" or "dry". Wet AMD occurs when abnormal new blood vessels form and grow into the retinal tissue. These new blood vessels can break and cause bleeding to surrounding tissue which can scar the retina. Dry AMD is characterised by small deposits called "drusen" forming in the retina. These drusen are made of debris from retinal cells, causing debris to build up at the back of the eye. This induces an inflammatory reaction that can damage the photoreceptors. There are many factors thought to be possible causes for macular degeneration. These are linked to age, gender, genetics, nutrition and exposure to smoking and sunlight.

2.2 Suggestions for Research Questions:

You can get some ideas by looking at one or two papers before you decide on your project. These are available online or pdf-files. This is only a small selection of available literature and more pdf-files or links to the library can be provided upon request

Age related macular degeneration and drusen: Neuroinflammation in the retina
Elisa Buschini, Antonio Piras, Raffaele Nuzzi b and Alessandro Vercelli a
Progress in Neurobiology 95 (2011) 14–25

Recent progress in understanding mammalian color vision
Gerald H. Jacobs
Ophthalmic Physiol Opt. 2010 30(5).

The expanding role of vascular endothelial growth factor inhibitors in ophthalmology.
Michael W. Stewart
Mayo Clin Proc. 2012 87(1):77-88.

The Retinal Pigment Epithelium in Visual Function
Olaf Strauss
Physiol Rev 2005 85: 845–881

Having read these you may think of some questions that you could investigate the retina. For example, you can look at the blood vessels and immune cells in the retina, using different preparations (whole mount or cross sections, which will be described to you at the start of the project). You can also look at the retina in different animals, for example the chicken or the pig and compare it to mouse and human (from the papers found in the library). You can also test the function of the eye in dark and light conditions, and look when you can see shapes and colours. You can investigate if age changes the function of the eye, by trying out simple test on your class mate, teacher, and grandparents.

In the first week of the project we will show you dissections of the eye and talk to you about the structure of the retina and the techniques we use to study it. In the following weeks you may try some experiments of your own to investigate the structure of the eye. The following is a plan of the kind of experiment you could do:

2.3 Study the different cell types of the retina in health and disease

Objective: Analyse different cell populations of the mouse retina.

Time required: Preparation, approximately 1 hour; running the experiment approximately 1 1/2 hours per day. This experiment will take 4 days to complete.

The research question: The retina consists of different cell layers including blood vessels and support cells (astrocytes and microglia). The blood vessels form a network to supply neurons with nutrients and oxygen. Blood vessels can be identified by cell specific markers, and one of these is collagen 4, expressed in the basement membrane of all blood vessels (see Figure 3 for an example). An alternative marker is CD31, which is expressed by endothelial cells, which form the inner layer of the vessel. In response to injury, new blood vessels are formed, which can be identified as sprouts growing in every direction. Vascular growth factor (VEGF) is a strong inducer of new blood vessels; this growth factor is released from RPE cells and macrophages in response to injury in the retina. Interestingly, antibodies against this growth factor are now being used in the clinic as a treatment for AMD. Muller cells and astrocytes are cells that provide support for neurons and mop up waste products. These cells are very sensitive to changes and alter their shape and number following injury, for example caused by inflammation or ageing.

The cells can be identified by a specific cellular marker, called glia fibrillar associated protein (GFAP, see figure 3). Another cell that responds to changes in the retina is the microglial cell, the immune cell of the eye. These cells are identified by the marker CD11b that increases after inflammation and injury.
In this project you will investigate the different cell types of a healthy mouse retina, using two different methods: a whole mount and cross sections of the retina. You will stain the tissue with antibodies that bind specifically to the different cells and use the microscope to analyse your results. The antibodies will be fluorescently labelled, and using different colours you will be able to see the blood vessels, the astrocytes and the microglia in the same tissue section. Using a technique called 'confocal microscopy' you will be able to take 3D images of your tissues.
Cross sections will also be stained with fluorescent antibodies, but you can also use a dye that binds to cell nuclei; this will enable you to see the different layers as seen in Figure 2 of the introduction.

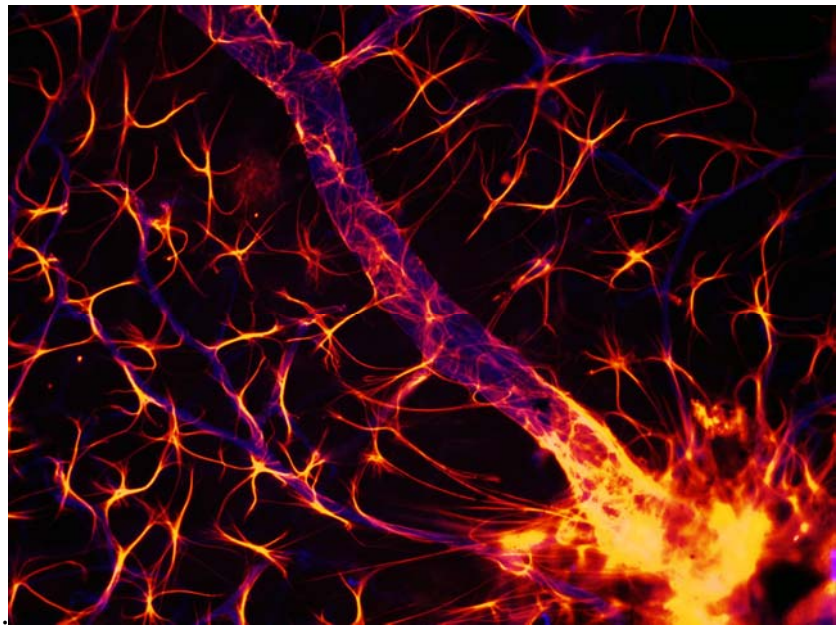

Figure 3: An example of a mouse retina stained for blood vessels and Muller cells. This experiment was performed at the University of Southampton by Salome Murinello.

Experimental protocol: You will stain a whole mount of a retina and cross section of the retina with different antibodies. The incubation of the tissue will take several days, and at the end of the week, you will mount the stained tissue to a glass slide and store it in the dark for analysis by the confocal microscope. This will be done at the University of Southampton, either in person or by video link.
During the incubation time you will use a dye to stain all nuclei to identify the different cell layer. We will also provide you with a simple test to measure black and white or colour vision under different

conditions. These experiments will enable you to better understand the function of the retina. We will also provide examples for stained section which you can use in the first week.

Equipment and resources:

Access to a binocular microscope with approximately x10 mag

Empty Bijous (for 7 ml)

1 ml eppendorfs

Plastic or glass Pasteur's pipettes

1 ml Gilson pipet with tips

100 ul Gilson pipette with tips

10 ul Gilson pipette with tips

Cross sections of mouse eyes

Whole mounts of retina floating in a bijous containing fixative

PBS tablets

Bovine serum albumin

Primary antibodies (rabbit-anti-mouse Collagen 4, mouse-anti-mouse GFAP, rat-anti-mouse CD11b)

Secondary antibodies (anti-mouse AF488, anti-rabbit AF568, anti-rat-AF350)

Aluminium foil

Glass slides

Cover slips

Mowiol Mounting medium (+/-Hoechst)

Hematoxylin and Eosine + alcohols and xylene

Method:

Staining whole mounts of mouse retina

Day 1(Monday)

Prepare PBS by adding one tablet/100 ml, then prepare 10% BSA solution by weighing out 10g/100ml. Make sure the solution is clear and all BSA is dissolved

Carefully remove the fluid from the bijous containing the retina's and replace with 5 ml PBS to wash the retina; you can use a Pasteur's pipette.

Repeat this washing step 2x

Remove all fluid from the bijous and replace with 1ml of freshly prepared PBS/10%BSA blocking buffer

Incubate overnight in the fridge

Day 2 (Tuesday)

Prepare fresh PBS by adding one tablet/100 ml.

Carefully remove the blocking buffer from the bijous containing the retina's and replace with 5 ml PBS to wash the retina; you can use a Pasteur's pipette.

Repeat this washing step 2x

Prepare your primary antibody by diluting the stock 1:1000 in 10% BSA. Make 1.5 ml of this solution.

Remove all fluid from the bijous and replace with 1ml of freshly prepared primary antibody solution

Incubate 2 days in the fridge

Day 4 (Thursday)

Prepare fresh PBS by adding one tablet/100 ml.

Carefully remove the primary antibody from the bijous containing the retina and replace with 5 ml PBS to wash the retina; you can use a Pasteur's pipette.

Repeat this washing step 2x

Prepare your secondary antibody by diluting the stock 1:1000 in 10% BSA. Make 1.5 ml of this solution.

Remove all fluid from the bijous and replace with 1ml of freshly prepared secondary antibody solution

Incubate 1 day in the fridge, cover your bijous with aluminium foil to protect against light

Day 5 (Friday)

Prepare fresh PBS by adding one tablet/100 ml.

Carefully remove the primary antibody from the bijous containing the retina and replace with 5 ml PBS to wash the retina; you can use a Pasteur's pipette.

Repeat this washing step 2x

Scoop the retina out using a paint brush, try not to rip and damage the tissue

Place the retina on a glass slide and carefully flatten it out

Mount the tissue with mounting medium (Mowiol) and cover with a glass coverslip

Keep mounted tissue in the dark in the fridge, until analysis by fluorescent microscope

Staining of cross sections

Day 1(Monday)

Use the freshly prepared PBS and blocking buffer from your whole mount experiment

Fill a glass vial with alcohol and place in the fridge for at least 30 minutes to cool to down to 4C. Place the glass slide with the cross sections of the tissue in the cold alcohol for 10 minutes. Carefully remove the glass slide from the alcohol and place into a glass vial filled with PBS. From this point forward it is important not to let the tissue dry out

H&E staining:

Place 1 glass slide with 5 retina's in hematoxyline solution (5 minutes), wash in tap water for 10 minutes, place in eosin (1 minute) and leave in tap water.

Dehydrate the tissue by placing in containers of 70% ethanol (5 minutes), 80% ethanol (5 minutes), 100% ethanol (5 minutes), fresh 100% ethanol (5 minutes), and leave in xylene (10 minutes)

Mount by placing generous amount of mounting medium (DEPX) on a glass cover slip and gently lay over your stained tissue. Leave in fuming hood for at least 1 hour before taking it out. Let the mounting medium dry completely before analysing under a microscope

Immunohistochemistry:

Place 1 rehydrate glass slide (with 5 retina's) in PBS

Circle around the tissue with a wax pen , or vaseline to create a barrier.

Place glass slide in a flat humidified incubating chamber, and immediately pipette 50 ul of PBS/10% BSA onto the tissue (you will see a dome of fluid within your wax circle, completely covering your tissue)

Incubate for 30 minutes at room temperature

Prepare your primary antibody solution by diluting 1:500 in PBS, make ~500 ul of this solution.

Optional: you can combine different antibodies for a double stain, and using multiple antibodies per retina, but make sure you also include a negative control (no primary antibody). We suggest you try single antibodies in week 1 and use combinations in week 2.

Carefully remove blocking buffer by gently tapping the glass slide onto tissue paper

Add 50 ul of primary antibody to your section

Incubate overnight in the fridge. Carefully transport your incubating chamber to make sure you do not move the glass slide

Day 2 (Tuesday)

Prepare fresh PBS by adding one tablet/100 ml.

Carefully remove the primary antibody by tapping the fluid off onto tissue paper

Place the glass slide into a rack and wash 3x 10 minutes in PBS

Prepare your secondary antibody by diluting the stock 1:1000 in PBS. Make 1ml of this solution

Add 50 ul of secondary fluorescent antibody to your section

Incubate 1 hour at room temperature

Carefully remove the secondary antibody by tapping fluid off on a tissue paper.

Place the glass slide into a rack and wash 3x 10 minutes in PBS

Mount by placing generous amount of mounting medium (Mowiol) onto your tissue and cover with a glass cover slip. Let dry the mounting medium dry completely before analysing under a microscope, and keep tissue in the fridge in the dark.

Suggestions for further study:

This assay could be modified to study the effects of other cells, such as endothelial cells or neurons. You can also investigate the anatomy of the eye of different animals, but dissecting the eye of a chicken or a pig and try to isolate the lens and retina. You could make drawings of different cell types of the retina after analysis of your tissue under the microscope, or create ART from your own results.

4. Resources required

Chemicals:

Phosphate Buffered Saline (PBS)

BSA (bovine serum albumin)

Absolute alcohol

Hematoxyline/eosin

Xylene

Primary antibodies (GFAP, Collagen 4)

Fluorescently labelled secondary antibodies (two different fluorescent colours)

Mowiol mounting medium, with Hoechst

Plastics:

7 ml bijoux pots

1 ml eppendorfs

Pasteur's pipettes

Glass slides

Cover slips

Equipment:

Confocal microscope

Binocular microscope

Gilson pipette (20µL, 100µL and 1000µL)

Gilson tips

Humidified chamber

Glass slide boxes

Other:

The tissue for this experiment is derived from waste products of ex-breeders.

Risk assessments for the procedures and reagents are provided.

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ExSite: EXperimental Science in ThE classroom: A project with the University of Southampton, funded by the Wellcome Trust Society Award in Authentic Biology