INSTITUTE OF OPHTHALMOLOGY

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Hey guys!

I took part in a 8-week internship at the Maldonald Lab. I worked with Dr Nicole to characterise the Killifish retina, verify antibodies and examine optimum protocol.

The African turquoise Killifish is a novel short-lived model organism. It's used to understand more about neurodegenerative diseases.

In this booklet we explore the importance of animal models (particularly Killifish). We will then explore the underlying mechanism behind visual transduction and the significance of photoreceptors. Followed by a basic introduction to 2 main experimental techniques: in-situ hybridisation (HCR) and immunohistochemistry (IHC).
CONTENT

ENGLAND IS AGEING pg 4

RETINAL DISEASES pg 5

FROM PATIENTS TO LABS TO CLINICAL TRIALS pg 7

SIGNIFICANCE OF ANIMAL MODELS pg 10

BIOGERONTOLOGY MODEL: KILLIFISH pg 12

LIFE CYCLE OF A KILLIFISH pg 15

TYPES OF RETINAL CELLS pg 19

VISUAL TRANSDUCTION pg 21

IMMUNOHISTOCHEMISTRY pg 25

IN-SITU HYBRIDISATION pg 27

WHATS NEXT? pg 32

SPECIAL THANKS pg 34
ENGLAND IS AGEING

Ageing is a significant risk factor for degeneration of the retina.

Some of the molecular mechanisms we see in other organisms underly humans. Hence animal models are a valuable source in understanding the bases of ageing.

We will look at the retina and models of retinal diseases - shown to blind the elderly population.

The effect of an ageing society has lead to a global public health concern. This is because of the retinal diseases that downstream lead to vision loss.

Ageing population in England 65+

2010: 9.2 million
2021: 11 million

(Office of National Statistics)

The population of England has continued to rise since 2011.

The population of elderly has risen from 9.2 million to 11 million.
RETINAL DISEASES

A prolonged life span means increased prevalence to age-related diseases...

AGE-RELATED DISEASES INCLUDE

NEURODEGENERATIVE DISEASES

ALZHEIMER'S

- Damaged Neurons
- Aggregation of Proteins

RETINAL DISEASES

AGE-RELATED MACULAR DEGENERATION

- Blur of the central vision due to damaged retina

GLAUCOMA

- Damage to optic nerves
- This leads to vision loss
The eye is an organ that translates light energy into chemical energy which is then transmitted to the CNS.

The image is projected reversed.
Early discovery of novel eye disease. Nobody knows anything about the disease or its underlying mechanism.

So what happens?

The clinics observe the disease and the effects of it. Recording symptoms and analysing trends in diagnosis.

While this happens, studies are being undertaken to understand more about the disease. We call these pre-clinical trials.

Pre-clinical trials are important because experiments help investigate cause of disease.

Scientists learn more about the biological pathway.
Experiments are carried in-vitro (outside a living organism) and in-vivo (inside a living organism).

In-vivo is an experiment that's performed on an animal (like fish) or humans.

In-vitro would look at a particular cell or a group of cells in a dish.

Successful results on animal models is published in the scientific community.

Some of these results are applied further and tested on human cells.... because we all know the human physiology is unique compared to animals.

Patient feedback:
- 5
- 4
- 3
- 2
- 1

Thank you for your work.
Scientific work is now approved for clinical trials. At this point, we are further investigating human diseases.

At this point, any approved drug or therapy is also tested for compatibility (only if approved by MHRA).

How are my patients coping?

Progression of disease?

Side effects?

Any physiological differences?

Significant improvement
ANIMAL MODELS

WHY DO WE LOOK AT ANIMALS?

Using animal models in research is controversial, but most results (working with them) have shown promising progression for diseases. That means treatments can be available sooner.
Species are chosen according to their genetic and functional characteristics to line of research e.g. killifish - to look at age-related retinal diseases.

Used to test for drugs, vaccines, genetherapy and immunotherapy

Understand pathology of disease in detail

Develop therapeutic interventions. This includes drug therapy, immunotherapy and gene therapy.

Toxicity?
Optimum dosage?
Side effects?
BIODERONTOLOGY MODELS

A SUCCESSFUL MODEL COVERS THE CHARACTERISTICS AND FUNCTIONAL CHANGES OF AGE-RELATED DISEASES...

BUT AT A RAPID PROGRESSION.

MODELS OF RETINAL DISEASES HAVE SHOWN TO IDENTIFY NEW SIGNALLING PATHWAYS INVOLVED IN THE INITIATION AND PROGRESSION OF DISEASE.

IF BOTH IN-VITRO AND IN-VIVO MODELS SHOW SUCCESSFUL RESULTS THE RESEARCH IS PROGRESSED INTO CLINICAL TRIALS.

THE PERFECT MODEL DOES NOT EXIST BECAUSE PATIENTS WITH DISEASES SUCH AS AND STILL EXHIBIT PHYSIOLOGICAL CHANGES.
THE AFRICAN TURQUOISE KILLIFISH IS A GREAT MODEL ORGANISM TO UNDERSTAND AGE-RELATED DISEASES. THEIR SHORT LIFE-Span OF 16-24 WEEKS. THE KILLIFISH CAN EXHIBIT VARIOUS PHENOTYPES IN SHORT PERIODS OF TIME. STUDIES HAVE SHOWN

"I CHOOSE YOU"

THE USE OF AGEING BIO-MARKERS CHARACTERISE AGE-RELATED PHENOTYPES. THIS INCLUDES MITOCHONDRIAL INSTABILITY, INCREASE IN APOPTOSIS, COLOUR LOSS AND 'VISION LOSS.'

2. The ponds are subjected to a brief rainy/wet season followed by a longer dry season.

* Diapause: Suspended development in killifish in unfavorable environments.
1. Juvenile Killifish

2. The eggs are ready to hatch. The golden/yellow eyes show when.

3. During the dry season, embryos enter diapause.


5. Growth is delayed to increase survival.

6. 16-24 weeks.
To understand what therapeutic treatment works best with patients suffering with vision loss, we need to characterise the characteristic and functional changes of retinal diseases.

Using killifish as a model organism to understand age-related diseases would be impactful. We still have a long way to go. Firstly, we need to characterise the retina of the killifish. We also need to find the best protocol e.g. antibodies that work well with killifish.

Understanding the structure of the retina and visual transduction is important because it only enhances our findings and learning.

This is the retina of the killifish. This is a killifish.
If we take the eye and cut it in half you get the cranial cross-section of the retina.

You can see the layers of the retina. Each is colour-coded to its abbreviations.

Each layer in the retina contain specialised cells. This is what we will look at next. The cross-section we will look at is shown above.
THE RETINAL EPITHELIUM LAYER (RPE) IS FOUND ON THE OUTER MOST SURFACE OF THE EYE, FOLLOWED BY THE PHOTORECEPTORS.

WE WILL FOCUS ON PHOTORECEPTORS IN DETAIL LATER ON.

LIGHT TRAVELS FROM THE RPE TO THE RETINAL GANGLION LAYER. WE WILL LOOK AT THIS NEXT.
**VISUAL TRANSDUCTION**

1. **First Light Passes Through Cornea:** Some of this light also enters the pupil.

   - **Visual Cortex**
   - **Lateral Geniculate Nucleus**

2. **The Cornea Allows the Light to Bend:** The iris controls how much light enters the eye.

3. **The Image Is Reversed:**
   - **Optic Disc**
   - **Optic Nerve**
   - **Blood Vessels**
   - **Nacula**
   - **Fovea**
   - **Retina**
   - **Iris**
   - **Cornea**
   - **Pupil**
   - **Lens**
   - **Ciliary Body**
2) Light hits the retina: the retina is packed with photoreceptor cells. These cells are specialised because they are light sensitive. The protein light pigments they contain make them unique (see table below).

They work by converging light signals into electrical signals. We will go into the details of this in the next page.

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### The Cone Has 4 Subtypes

The table below shows the protein pigments.

<table>
<thead>
<tr>
<th>Pigment Molecule</th>
<th>Rods</th>
<th>Cones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rods</td>
<td>Rhodopsin</td>
<td></td>
</tr>
<tr>
<td>Cones</td>
<td>Blue cone, red cone, UV cone, green cone</td>
<td>Blue opsin, red opsin, UV opsin, green opsin</td>
</tr>
</tbody>
</table>
The light is absorbed by the photopigments found in photoreceptor discs. These are found in the inner segment.

1. The visual pigments are embedded into the membranous disc.
2. The light absorbed triggers a conformational change.
3. This initiates a series of events that trigger a potential.

Light absorbed here!

A bunch of other proteins that facilitate the chain reaction

The depolarisation closes the sodium channel. This hyper-polarises the cell.

Depolarisation

Secretion of vesicles
Our objectives for this experiment was to:


These were performed on 4-12 week killifish.

From this we were able to:

- Assess genetic component of photoreceptor opsin genes.
- Visualise the localisation of different genetic probes in different retinal cell-types.

Our focus will be on photoreceptors because diseases caused by vision loss is common in age-related diseases.

In the next few pages we will go through experimental techniques including IHC and HCR.
IMMUNOHISTO

Tissue slides stored in freezer

Chosen slides left to settle @ room temp

Inubated with primary then secondary antibodies

Rehydration & fixation of cells preserves integrity of tissue

Blocking serum prevents non-specific binding

If this is the photoreceptor we are interested in visual...

We need to make the cell more permeable to allow antibodies to penetrate and react with photoreceptor pigments.

Kiwi fish retina tissue

Treated with antigen retrieval

Primary antibodies (1)

Secondary antibodies (2)
## Chemistry

Addition of a 1' Antibody (binds to multiple rhodopsin):

- **ZPR3**
- **ZPR1**

Addition of 2 antibodies (binds to the 1' antibody):

- **UV-Opsin**
- **Blue-Opsin**

### IHC Rounds

<table>
<thead>
<tr>
<th>Rounds</th>
<th>Antibodies Used</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Round One</strong></td>
<td>ZPR1</td>
<td>Double red/green cones</td>
</tr>
<tr>
<td><strong>Round Two</strong></td>
<td>ZPR3</td>
<td>Rhodopsin in rods and green cones</td>
</tr>
<tr>
<td><strong>Round Three</strong></td>
<td>UV-Opsin</td>
<td>UV cones</td>
</tr>
<tr>
<td></td>
<td>Blue-Opsin</td>
<td>Blue cones</td>
</tr>
<tr>
<td><strong>(Different Protocol)</strong></td>
<td>UV-Opsin and ZPR1</td>
<td>Blue Opsin and ZPR3</td>
</tr>
</tbody>
</table>
HCR

In situ hybridisation (also known as HCR) allows us to study gene expression and gene product. In particular those that we are interested in:

- What does it do?
- How is this protein regulated?
- How does it behave in disease?

All these information provide us with information on phenotypic function and gene regulation.

HCR on killifish have shown high specificity and single molecule sensitivity.

So, how does a HCR work?

The mRNA of interest is imaged. HCR works by amplifying mRNA expression - to boost fluorescence signaling.
Our aim with in-situ hybridisation (HCR) is to visualise specific cell types in the retina. This is different from immunohistochemistry because it targets mRNA.

Inside the protein factory:
- Ribosomes
- mRNA strand
- Nucleotides
- Opin proteins

What it looks like with HCR:
- This amplifies molecular signalling (more detectable)
- Multiplexed mRNA signalling in tissue mounts or whole mount killifish gives us distinct fluorophore imaging
1. **Hybridise Probe Set and Wash**

   - The split initiator probes each carry an initiator. This strand then binds onto the targeted mRNA.
   - Any unused probes are washed away.

2. **Specific Hybridisation**

   - These split initiator RNA probes hybridise to mRNA specifically!

3. **In Turn Hybridising**

   - The hairpins drive the assembly cascade to an RNA initiator sequence.

4. **In Turn Hybridising**

   - In turn hybridising into the input domain of H2.
1. Hybridisation of H1 to initiator leaves the H1 output domain exposed.

2. Specifically bound probes trigger the self-assembly of fluorescent amplification polymers (the hair-pins labelled as H1 and H2)

3. Any unused hairpins are washed away.
The RNA probes we looked at are listed below on a table. 

Don’t forget, these are RNA probes we are interested in.

The RNA probes can also be called initiators.

<table>
<thead>
<tr>
<th>INITIATOR</th>
<th>BIPOLAR CELLS</th>
<th>MICROGLIAL</th>
<th>PHOTORECEPTORS</th>
<th>HORIZONTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>eIna</td>
<td>nph2</td>
<td>olx2</td>
<td>metap2</td>
</tr>
<tr>
<td>B2</td>
<td>Fez1</td>
<td>neurod1</td>
<td>Pax6b</td>
<td>mHox4</td>
</tr>
<tr>
<td>B3</td>
<td>Sebox</td>
<td>dim3b</td>
<td>nph2</td>
<td>Sk7a2</td>
</tr>
</tbody>
</table>

This was compared to a control of probes (that have shown to work well in previous studies).

Each of these probes play a significant role in the retina.

However, for now we are only interested in visualising it.
There are many directions we can take to understand retinal diseases using killifish as fish models is one of them as well as perfecting protocol.

The age of onset influences management of disease.

Hence I would look at the retinal genes in different killifish age groups.

What more?

- We will challenge and trial various protocol to achieve best results.
- Try various other antibodies (specific to photoreceptors) and see how they work in healthy and diseased killifish.
- Develop therapeutic interventions to treat retinal diseases.
SPECIAL THANKS

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