Home Office

NON-TECHNICAL SUMMARY

Multi-tissue mechanics in the development and engineering of the posterior body axis

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

morphogenesis, differentiation, patterning, mechanics, stem cells

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Vertebrate embryos have a very similar layout of our major organs: a central nervous system, a segmented vertebral column and associates muscles and a central digestive tract. This 'body plan' is established early in development and is remarkably reproducible between individual embryos, and between species. This project aims to understand the principles underlying such robustness so that we can copy the same approach and improve the creation of tissues from embryonic stem cells in the petri dish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project aims to reveal some fundamental principles about how cells make collective decisions during early development to become specific cell types. In healthy situations, cells will retain these specific cell types throughout an organism's entire lifetime. However, in disease states such as cancer, cells reverse the programming received during development and re-enter a state that leads them to divide excessively, generate alternate cell types, or migrate into the body and establish new tumours elsewhere. Therefore, the more that we understand the fundamental process involved in development, the better we can understand what goes wrong in cancer. Stem cells are cells that, like very early embryonic cells, can generate all cell types of the body. Recent work has enabled researchers to generate stem cells from cells of adults, that can then be redirected to many other different cell types. A major hope for the treatment of many diseases is through the use of stem cells to generate replacement cells, tissues or even organs. In addition, the ability to generate multiple cell types from an individual patient is revolutionising the study of disease and opening up the possibility of patient-specific medicine. For any of these possibilities to realise their full potential, we need to direct stem cells in a very well controlled way, without the risk of cells taking decisions that would be harmful such as entering a cancer-like cell state. By investigating the fundamental processes coordinating cell fate decisions and movement in early development, we hope to inform researchers working in the development of stem cell differentiation protocols and regenerative medicine. For this reason, we directly collaborate with stem cell researchers on a number of different projects and even have some in the lab.

What outputs do you think you will see at the end of this project?

We expect to gain a better understanding of how two key processes that operate during embryonic development work together. The first relates to cell movements, and the mechanical impact cell populations have on one another. The second relates to gene expression changes, and how signals alter this as cells move through the embryo. This will result in new conceptual advances that will impact the design of strategies to drive the differentiation of stem cells in culture. It will result in a series of publications in academic journals. We will ensure to communicate all outcomes of our work including both successful and non-successful approaches.

Who or what will benefit from these outputs, and how?

Short term: academics in a similar field will be informed of our research through conferences, pre-prints and peer-reviewed publications.

Mid term: academic researchers in the stem cell field will adopt new strategies for targeted cell differentiation

Long term: adoption of improved stem cell protocols in clinical and industrial settings.

How will you look to maximise the outputs of this work?

We are active in the Open Research community, choosing to make all our results open to the community as fast as possible. We also seek to openly share our live imaging datasets with the community where possible. Any improvements in protocols will be rapidly communicated using forums such as F1000 and ZFIN.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 14400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

During the development of the embryo, cells have to decide on becoming all the different cell types that make up the adult body. They also have to do this in a very coordinated way, with tens of thousands of cells all deciding which part of the body they are going to generate. How this coordination is achieved is one of the great mysteries of biology. Our current understanding is based on the idea that long-range signals travel across the embryo to instruct cells about which cell type to become. Cells close to the source of the signal would receive a lot of it and therefore turn into one cell type, while those further away would receive less and therefore decide on a different fate. However, this only works in some cases, as the cells themselves are moving around very rapidly. Imagine a headmaster shouting instructions to an entire school of children playing outside. Whatever initial pattern that might be set up is going to be rapidly destroyed as they move around the playing field.

Another way is to make use of the fact that cells travel within distinct cell layers and are constrained by mechanical forces to move in streams. It's a bit like having the children back inside the school moving between different classrooms. Now the teacher can move through the corridors and give out specific instructions as the children move past. In the same way, specific signalling cells move through the embryo and pass on instructions to other cells as they move past. In reality, many teachers are required to walk the corridors of the school to make sure everything is kept in check. Note that in this

model, it's the timing of when pupils meet the teachers that will determine how patterns form, rather than their spatial position as in the playground idea.

To understand this complex process, we collect information from each cell and its state (or each pupil and whether or not they have their shirt hanging out!) and track their movements as they move through the embryo. At the same time, we collect information about the instructions each cell is receiving. This is a huge amount of information that requires computer power to simulate the outcome. But once this is achieved, we can ask questions about what the minimal set of instructions are that are required to generate a well-coordinated pattern of cell differentiation.

Zebrafish embryos are ideal for this sort of large-scale experiment, for several reasons. Firstly, their embryos are visually transparent, meaning that we can image all the cells in the embryo at once. Secondly, they develop externally outside of the mother and require only a simple temperature control for them to develop normally, meaning that we can culture them under a range of different microscopes and follow their development by time-lapse microscopy (a series of images taken of embryo development that allow for the generation of movies showing cells in action). Finally, we are able to insert new DNA sequences into their genomes that make fluorescent proteins which light up when certain genes are being switched on or off, allowing us to follow where specific signals are being received by cells as they travel through the embryo. Together, this means we can collect all the information required to watch how patterns in cell type differentiation occur in real-time, and across the whole embryo.

The outcome of this project is a better understanding of how normal development happens during the formation of the vertebrate body plan. Also, it provides new ideas about how we might control the differentiation and morphogenesis (the shaping of tissues and organs) with of stem cells cultured in the dish.

Typically, what will be done to an animal used in your project?

The adult zebrafish will be housed in a dedicated aquarium within the department, run by trained staff. We will generate genetically altered zebrafish by introducing modified genetic material at the 1 cell embryo stage and growing these embryos to adulthood. In order to know which fish contain genetic alterations we sometimes need to carry out genetic analysis via e.g. cutting a small portion of the fish's tail fin under general anaesthetic and analyse the genetic code inside this tissue. The fish is then kept in a separate tank with fresh water and the fin then regrows relatively quickly (within approximately 2 weeks). Once the fish have recovered from the anaestic they swim and behave normally, behaviour is monitored regularly by facility staff. Where appropriate, other methods of genotyping may be used, such as swabbing the surface layer of the skin. Adult fish will be maintained until a maximum of 30 months of age (although we aim to only keep adult fish until 18 months of age in the majority of cases). During this time, adult fish will be bred in specialised breeding tanks to enable the production of genetically altered zebrafish embryos. We very occasionally need to anaesthetise fish for the collection of eggs and sperm. At the end of the protocols fish will be humanely killed or supplied to other project licences or recognised establishments with the authority to breed and maintain genetically altered zebrafish of this type.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not usually expect there to be adverse effects to adults caused by the genetic alterations that we introduce. However, for example in the case of introducing optogenetic genes (a region of DNA that codes for proteins that can be activated and inactivated upon exposure to light of specific wavelengths), it is sometimes possible that some adverse effects might arise in the fish. If this occurs, we would expect such effects to be mild (such as thinner bodies). However, it is possible that moderate effects might occasionally arise (such as significantly bent body-axis, which might effect swimming). It is also possible that the survival of larvae to adulthood might not be as high in some genetically modified lines when compared to wild type lines. If moderate effects were to occur, we will humanely kill the affected fish. We do not expect there to be any adverse effects from breeding the zebrafish. It is unlikely but possible that fish might develop an infection following removal of a small part of the tail fin, in which case we will humanely kill the fish. For both genotyping and sperm/egg collection, it is possible that fish may not recover from anaesthesia but this is very unusual (less than 1%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish. Mild: 90%. Moderate: 10%

What will happen to animals at the end of this project?

- Used in other projects
- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The central aim of the project is to follow cells as they make decisions in their normal environment. Therefore, these experiments can only be performed in an animal. However, all experiments are performed at an early stage of life development (zebrafish embryos younger than 5 days old) which do not require protection in law.

Which non-animal alternatives did you consider for use in this project?

We have introduced the use of embryonic organoids in our lab, derived from mouse embryonic stem cells. In addition, we make use of mathematical modelling to more rapidly explore mechanisms of development and better refine our hypotheses- that ultimately reduces animal use. Stem cell biology and regenerative medicine is currently undergoing a major shift towards the increased use of organoids- these are 3D multi-cellular aggregates that are allowed to grow and develop in a culture

dish (in vitro). While they can make many of the specific cell types of a multitude of different organs, the look and shape of these structures are usually very variable and often do not look like what is generated during normal development. We take the lessons learned from our understanding of multi-tissue development in zebrafish and use this to design new ways to better control and engineer the development of similar structures generated from mouse embryonic stem cells, called gastruloids.

As a rule of thumb, we like to let cells run their own, unmanipulated genetic programmes. We also let them talk to one another using signals that they normally would if they were in an embryo. Our central hypothesis is that by leaving these genetic and molecular components alone, we can refine development in a controlled way, simply by changing the external mechanical environment of the gastruloids. This would be like moving and shaping the school corridors, without changing the instructions shouted by teachers, or the ability of the pupils to listen to them. Instead, we act like a group of sheep-dogs corralling sheep into a tight group and guiding them to where they need to be. However, to become effective sheep dogs, we need to know how this is achieved in normal development first and this is where our work with zebrafish embryos comes into play.

An additional way to replace a large number of animals for scientific research, is to make use of mathematical models and computer simulations to ask whether a certain set of observations are sufficient to generate the biological process we are interested in. This helps us to define more precisely the experiments that are most scientifically interesting and of relevance to perform in the embryos on this project and will ultimately reduce the number of animals required for the project.

Why were they not suitable?

The central aim of the project is to understand how gene expression patterns are generated by cells as they go through the normal cell movements associated with embryonic development. While in vitro models of development can recapitulate multiple aspects of patterning, they do not acheive this with the same reproducibility and robustness that is seen in normal embryos. Our lab is set up to use zebrafish embryos where we can learn how this is achieved in normal development, and then transfer the lessons learned to mammalian embryonic organoids. Overall, it is a complementary approach.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All of our experimental work will be carried out in zebrafish embryos younger than 5 days old (which are not protected under The Animals (Scientific Procedures) Act 1986. Animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. The number of

adult animals used is therefore solely related to the numbers required to maintain sufficient breeding stocks of animals.

Animal usages was based on requiring approximately 120 fish per new generation of fish for each genetically modified line. We will make a new generation for each line every year (5 generations per line). In addition, when generating new genetically modified lines, the F0 embryos need to be genotypically screened. Therefore, approximately 200 additional fish may be required per new line at the F0 stage to find appropriate founders to generate the F1 generation. Based on raising 15 established lines and generating 4 new genetically modified lines over the course of the 5 years, this makes approximately 10,000 adult fish. In addition, we have listed a maximum number of 2000 of these fish that might be kept on an alternative breeding protocol, depending on their genetic background.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will carefully design our experiments so that we use appropriate numbers of embryos for each experiment. Where necessary and possible, we will carry out pilot studies to determine the number of embryos required to achieve robust statistical analysis. If we require assistance in our experimental design, we will make use of available guidelines and online tools such as the NC3Rs EDA (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) and PREPARE guidelines (https://norecopa.no/more-resources/experimental-design-and-reporting/). We will ensure that our publications conform to the ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

To make our experiments robust, we will control for variability in the following ways:

We will reduce environmental variability by carefully housing breeding adult fish in the dedicated zebrafish facility and by keeping genetic background constant within each genetically modified line of fish.

We will assess normal levels of variability within experiments via pilot experiments, allowing us to select appropriate statistical methods and number of embryos.

We will reduce bias by randomly selecting embryos collected from a pool of breeding adults and, when possible, by assigning treatment and control groups in a way that is unknown to the person analysing the data (blinding).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are using several methods to reduce the numbers of adult animals used. First, we will share relevant fish stocks with other users within the facility. Second, we will try to limit repeated breeding to once per week to optimise breeding performance. Third, we will minimize the generation of embryos wherever possible for our experiments. Fourth, we will freeze sperm from genetically altered lines of zebrafish for longer-term storage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use the zebrafish because (1) its anatomy and genetics are a good model for other vertebrate species; (2) its embryos are externally fertilized and can be obtained without harm to the mother; (3) they are large and near transparent, facilitating imaging studies.

We don't envisage any suffering in licensed animals beyond the mild procedures described above. We will only use zebrafish embryos younger than 5 days old for our experiments, which are not yet capable of independent feeding or complex cognitive behaviours. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments and, where possible, by anaesthetising embryos that are sufficiently developed to be capable of initiating movement during imaging (those above 18 hours old).

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be optimised and carefully controlled.

We have refined our fin clipping to remove only a very small region of the fin, that alleviates the need to provide analgesia.

Why can't you use animals that are less sentient?

It is essential that we use a vertebrate model for our work, as we want our results to translate to the manipulation of mammalian stem cells in culture. The zebrafish embryo is the most refined vertebrate model possible for the work that we propose. The zebrafish embryo is also an ideal model system for studying organ development, since they are small, transparent, develop rapidly and it is possible to alter their genetics in a reasonably straight-forward way.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We don't envisage any suffering in the vast majority of licenced animals beyond the mild procedures described above. Moderate effects might occasionally arise due to the genetic alteration of the fish, as described above. If that occurs, the animals will be promptly killed using a humane method. We will only use zebrafish embryos younger than 5 days old for our experiments, which are not yet capable of independent feeding. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments and, where possible, by

anaesthetising embryos that are sufficiently developed to be capable of initiating movement during imaging (those above 18 hours old).

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be carefully controlled.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Appropriate experimental design for our experiments in unlicenced embryos under 5 d.p.f. will be carried out, as described in the 'Reduction' section above. Licenced animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. These will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time trained animal technicians, who will ensure their welfare, in line with their training on best practise. There are several resources to inform us about the current research on refinement of procedures (e.g. https://norecopa.no/species/fish/, https://nc3rs.org.uk/3rs-resources/zebrafish-welfare, https://www.lasa.co.uk/current_publications/). These will be taken into account when deciding on the most appropriate method for procedures..

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in 3R tools are internally circulated. We can also access advances via the NC3Rs (https://nc3rs.org.uk/resource-hubs) and Norecopa website pages (https://norecopa.no/databases-guidelines). If scientifically appropriate advances in 3Rs arise in the course of the project, we will seek advice from the named veterinary surgeon and named animal care and welfare officer about whether and how to implement them.