Home Office

NON-TECHNICAL SUMMARY

In vivo compressive forces

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

cell migration, cancer, nucleus, mitosis, mechanics

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?

To use Zebrafish as a model, relevant to human disease, to study in vivo mechanical stress.

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?

When they divide or migrate, cells in tissues have to push against other cells around them or squeeze through very tight spaces.

Studies in cell culture where cells were forced into very small spaces, such as very small channels made of silicone (microchannels), showed that single cells that are physically compressed experience mechanical stress. This can lead to DNA damage because of deformation and rupture of the cell nucleus or to errors during cell division. As a consequence, mechanical compression can also cause changes in the DNA of cancer cells which can be inherited and may contribute to cancer.

However, whether *in vivo* (in an intact animal or tissue) compressive forces can affect cell divisions, cell differentiation or cause heritable DNA damage has not been yet investigated. Indeed, it is challenging to image migrating cells in confined environments in mammalian tissues as these need to be surgically exposed. Moreover, it is difficult to manipulate the physical properties of tissues in vivo.

My research team and I will overcome these challenges by using the Zebrafish. As its embryo is small and transparent, we will be able to visualise migrating cells directly in an intact animal that is suitable to mechanical manipulations. We will investigate a population of embryonic multipotent stem cells (cells of the embryo that can differentiate into many different tissues) called neural crest cells. These cells are essential to development of all vertebrates, and differentiate into many kinds of tissues, including cartilage and bone, pigment cells of the skin, neurons and glia. In the trunk of the Zebrafish embryo, we observed that neural crest cells migrate through a narrow space in between other tissues before differentiating into glial cells and neurons. This confined migration is highly conserved across vertebrates, including humans. We have observed that neural crest cells experience significant nuclear deformation during migration. Moreover, they divide asymmetrically, giving rise to daughters with different fates, and, importantly, sometimes they make errors when dividing.

These findings are important for human health, because trunk neural crest can initiate neuroblastoma, a common solid tumour in children. Neuroblastoma still has only 50% survival rate and in 98% of cases is linked to chromosomal abnormalities rather than germline mutations. It is unknown why neuroblastomas suffer genomic errors. One possibility is that this might be a consequence of in vivo mechanical stress.

Using the Zebrafish as a model we will dissect, using a multi-scale approach, whether physiological mechanical compression can cause DNA damage in cells within a developing vertebrate embryo. Our work will elucidate whether physical stress can contribute to human cancer initiation, and will shed light on the origin of chromosome damage in cancer.

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

The proposed research aims at understanding whether mechanical stress occurring during *in vivo* embryonic development contributes to DNA damage and mitotic errors, and whether such mechanical inputs could contribute to cancer initiation *in vivo.*

This project will be transformative for a field that has so far heavily relied on in vitro microfabrication approaches to understand the consequences of mechanical stresses on cell function (i.e. nuclear integrity, mitosis, cytoskeletal organisation, cell fate specification), and the publication of findings in a physiological in vivo model such as Zebrafish neural crest cell migration will finally address this gap.

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

The immediate beneficiaries of the proposed research will be other researchers working in the UK and internationally in the fields of tissue mechanics, cell migration, developmental biology, cancer cell biology. My research will pioneer novel strategies to manipulate tissue forces in vivo in Zebrafish that will be useful to other researchers working in the field of developmental mechanics. My collaborators will directly benefit from the research 1) by co-authoring the scientific publications that will result from it; 2) by exploiting any relevant findings for further investigations in the future.

In addition, the implications of our research will be relevant to clinical and non-clinical researchers studying cancer initiation , metastasis, potential therapeutic targets and drug resistance mechanisms, as well as other key stakeholders: (a) clinicians, healthcare professionals and public policy makers; (b) pharmaceutical companies looking to translate findings; (c) students and researchers receiving lab experience and training in skills in in vivo research; and (d) the lay public through outreach initiatives.

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

Before publication, the results of the research will be discussed with the research community at specialised national and international in-person and online conferences as well at local seminar series.

The results of our research will be published in the appropriate specialized journals. Upon submission to journals, articles will be made available to the scientific community as preprints by uploading them to preprint servers . Upon publication, papers will be advertised to their readership via official press releases, via community websites and on social media.

Moreover, my laboratory will also generate novel genetic tools and materials that will be made publicly available upon publication (or earlier, upon reasonable request) and will shared with other researchers via community resources.

My research team and I also believe that engaging with the non-academic public is an essential part of the work of a researcher. While publishing work in specialised journal benefits the science community, it is important that the wider public understands and trusts the work we undertake as scientists. We intend to keep engaging with the local community to ensure the implications of our research are well understood by the wider public.

For example, my lab will participate at science festivals. We plan to develop activities that help non-academic audiences understand what the consequences of mechanical stress on cells are, and why is it important that we understand this to fight cancer.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 12,008

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.

We will use zebrafish embryos for this research because they are transparent, develop rapidly and we can carry out genetic and mechanical manipulations in these small embryos. The neural crest cells are formed over approximately 24 hours. This means that we can put the intact embryo under a specialised microscope and can easily image the whole process of vertebrate neural crest migration within the course of one experiment. Neural crest cells are found in all vertebrates and the mechanisms of neural crest migration are conserved in humans. All of our experiments will be carried out on embryos under 5 days old, which do not require a licence from the Home Office. To generate these embryos, it is necessary to generate and maintain a breeding population of adult fish, which does require a licence.

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 fin clipping: 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). 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 18 months of age. When possible, healthy fish may be maintained until 30 month of age on a specific ageing protocol to reduce the number of animals used in the project. 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 fluorescent reporters for subcellular components, it is sometimes possible that, due to basal activity of these genes, some adverse effects might arise in the fish. If this occurs, we would expect such effects to be mild (such as thinner bodies, mild lordosis that does not affect swimming). However, it is possible that sometimes moderate effects

might occasionally arise (such as significantly bent body-axis, which might affect 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. 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: 85%. Moderate: 15%

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

- Killed
- Used in other projects
- 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 focus of this project is understanding how cells cope with mechanical stresses when squeezing in small spaces inside the body of a living animal. It is important to use an animal rather than looking at cells in culture because the physical properties of cells in a dish and the interactions between cells and their environment are very different in a culture system. Therefore, it is only possible to fully understand the cellular responses to mechanical stresses by looking inside an intact animal. However, we are able to carry out some of our research using cells grown outside an animal (cell culture) – for example we have recently started a collaboration with another team of researchers to understand how human neural crest cells derived from embryonic stem cells cope with being squeezed in a dish and have accordingly reduced the number of animals used in our research. As we investigate the animal model alongside this culture system, we will learn whether more of our work can be carried out in culture.

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

We have introduced the use of cell culture models in our lab, which we will use in the near future in collaboration with another team of researchers to replace some of our animal experiments. For example, our animal experiments will allow us to measure the size and shape of the spaces where neural crest migrate inside the embryonic animal tissue. We will use microfluidics to generate channels

of similar size and shape and investigate how cultured cells respond to compression in these small spaces. We estimate that carrying out this work in culture will reduce our anticipated animal usage by roughly 20%.

Why were they not suitable?

It is not possible to entirely replace animal experiments for this project since it is important to test whether the principles identified in cell culture are the same *in vivo*. This is especially important since the main aim of this project is understanding the consequences of mechanical stress on migrating and dividing cells *in vivo*, which will be dependent on the physical properties of the tissues surrounding our cells of interest.

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?

Most of our experiments will be carried out on embryos younger than 5 days post fertilization (dpf), 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.

For this reason, the numbers of zebrafish necessary to carry out the research we are proposing will only depend on the number of Zebrafish that are required for breeding. For a robust stock we maintain approximately 2 tanks of 20 fish per transgenic/mutant line with a 50:50 male to female ratio (40 fish/line). For each stock a new generation will be grown every 18 months (3.3 generations per line in 5 years). Because sometimes most fish in a tank will end up being of the same sex and this is difficult to control, it often can result in a gender bias, so we will raise double the number of fish that will be eventually maintained (80/line).

We will maintain 20 existing transgenic lines and two wild type lines and generate ten new transgenic lines (80x32 lines=2560 fish). For each we will breed a new generation every 18 months (2560x3.3=8448). When generating new lines, the F0 embryos will need genotyping: approximately 100 additional fish will be required per new line at the F0 stage to find founders for the F1(100 x 10= 1000) for a total of 2560+8448+1000=12008 fish.

However, we anticipate that the numbers that we will actually use should be significantly lower, since we are committed to actively reducing animal usage (see below).

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

Animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. Therefore, it is not possible to reduce the number of licenced animals used via experimental design.

However, we still aim to reduce the number of unlicenced zebrafish embryos under 5 days old that we will use in our experiments via careful experimental design. There are several experimental design assistants and guidelines available to help us with appropriate design of each experiment. For example, 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 also ensure that our publications conform to the ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

For example, 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 will use several strategies 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 the number of new generations being grown for each line by maintaining fish up to the age of 30 months when possible. Third, we will minimize the generation of transgenic lines and use wild type embryos wherever possible for our experiments. Fourth, we will freeze sperm from genetically altered lines of zebrafish for longer-term storage. We will also carry out efficient genotyping and raise fewer fish per generation wherever possible.

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.

For this project we will use Zebrafish to establish and maintain breeding stocks of fish that will be used to produce genetically altered embryos. Because the Zebrafish embryo is small and translucent, it is especially suited to investigate how cells dynamically respond to mechanical stresses in an intact animal.

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

The key aspect of our programme is to visualise and perturb neural crest cells behaviours in situ by advanced microscopy techniques or micromanipulation. We use the Zebrafish because it offers unique advantages for imaging studies due to its optical transparency: in contrast with mammalian tissue, Zebrafish embryos can be imaged in a completely non-invasive manner.

Since neural crest are an evolutionary feature of vertebrates, to investigate neural crest cell behaviours it is necessary to use a vertebrate model system. The zebrafish embryo is also an ideal model system because they are small, transparent, develop rapidly and it is possible to alter their genetics in a 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. If that occurs, the animals will be killed using a humane method as soon as possible. In certain occasions, for example if a fish is the founder of a new transgenic line and develops moderate effects, it will be killed as soon as the next generation reached breeding age and bred successfully, to minimise suffering without hindering the progress of the project. 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 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. Environmental enrichment is currently provided to the fish by feeding them live invertebrate foods, providing them with a small current of water they can swim against and occasionally by enriching the environment with plastic plants.

Whenever fish may have to be maintained in tanks in small numbers (i.e. when transgenic founder fish are identified) they will be housed with companion fish that can be distinguished by their body pigmentation or be housed in a tank with environmental enrichment.

If a transgenic line is not being regularly used, it will be preserved by sperm freezing to ensure that no unnecessary numbers of animals are generated.

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 practice. We will make use of available 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.