Home Office

NON-TECHNICAL SUMMARY

Using zebrafish to understand neurodegenerative disease and develop therapies

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

zebrafish, disability, mechanism, therapy, microbiome

Animal types	Life stages
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult

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?

This project aims to increase our understanding of diseases and the functions of the mutated proteins that cause those diseases, as well as providing disease models for therapeutic discovery and development.

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?

The project focuses on rare inherited diseases, which together affect about one in every 10 people, even though each disease has an incidence of less than 1 in 2000 people. They frequently cause premature death in childhood and the vast majority have no approved treatments. We will also focus on epilepsies, which affect 9.37 per 1000 people in the UK. 30% of these patients do not have their seizures adequately controlled which causes reduced quality of life, so new treatments are needed. We will also study diseases that have a more complex origin, in which the same genetic factors interact with environmental factors. There is a huge need to find treatments for these diseases. As the genetic diseases we study affect several organs, and all cells are exposed to the treatments we are testing, live animals are required.

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

Through the data we generate, we expect to have increased our understanding of the function of two or more proteins involved in childhood neurodegeneration and epilepsy, including understanding how those proteins interact with other processes within the cell and how disease arises. We will focus our studies, but not limit them, to CLN2, CLN3, CLN7 and CLCN4 disease, caused by mutations in CLN2, CLN3, CLN7 and CLCN4 proteins. This will also inform our understanding of complex diseases where these proteins are involved.

We plan to generate several disease models, and by studying them we will better understand the disease process.

Several chemicals will be tested. The data will indicate to us if they are likely to treat any of the diseases that we are studying.

We will determine whether alterations to the body's bacteria affect the severity of the disease we are studying.

We will publish our findings and present them at conferences.

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

In the short-term scientists will benefit from increased understanding of childhood neurodegenerative diseases and epilepsy, including understanding the normal function of the protein that is mutated in the disease, and what goes wrong in the cells when the protein is mutated. This will lead to better treatments for patients in the long term. This will also increase our understanding of related complex diseases.

The availability of our disease models will enable treatments to be tested on more model species so that everyone can be more confident that the treatment will work on patients, who will benefit in the medium term.

The zebrafish develops very quickly and most of our models show the disease at just a few days old. As they are so small, our zebrafish disease models can be used to test thousands of chemicals in a relatively short amount of time, leading to treatments getting to patients earlier. This will benefit scientists in the short-term and patients in the long-term.

The identification of chemicals that can be developed into novel drug treatments for childhood degenerative diseases and epilepsies and the knowledge of how the chemicals work to combat the disease will support efforts to bring the compound to the clinic in the medium to long term.

Understanding the contribution of the body's bacteria to childhood neurodegenerative diseases and epilepsy could result in advice to improve patients' symptoms in the medium term.

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

We will usually publish our work as open access after completing a body of research, however it is difficult to publish results showing that a treatment does not work. An alternative is to publish a Registered Report. Registered Reports are where we publish our planned experiments before we actually do them and are guaranteed that the results will be published not matter what the results show, even if the treatment does not work. As long as it does not negatively affect our chance to protect our intellectual property via patents, experiments that test to see if chemicals can treat the disease will be published as Registered Reports.

We will disseminate our research at international conferences.

We collaborate with others using different models or methods to extend the impact of, and increase the confidence in, our research.

We will protect the Intellectual Property generated through filing patents, where appropriate.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 21400

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.

Data from zebrafish can be extrapolated to humans due to a high level of similarity, particularly in the basic chemical and cellular processes. Responses to chemicals and drugs are also similar. This means that it is valid to use the zebrafish to model disease, investigate what is going wrong in that disease, and search for and test novel treatments. The majority of the disease models we use are for inherited neurodegenerative diseases and epilepsy, so in this case we also know how similar the gene and the protein that is made from it is between zebrafish and humans. We can also manipulate the bacteria of zebrafish in several ways.

The majority of our experiments will be performed on developing zebrafish embryos and larvae before they are considered animals that are regulated by the Home Office (which is 5 days old). Zebrafish are embryos until about 3 days old, when they become larvae. This means we are using a replacement animal for our experiments. We can do this because the disease can be seen in the developing embryonic and larval zebrafish as they develop rapidly and they are visible as their development is external to the mother. This also means the mother is not harmed. Thus, the zebrafish is also the most refined whole animal model for our research.

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

We will study existing and new disease models, which we will create. To create new models, we inject the one cell zebrafish with agents to specifically alter a targeted gene. We select healthy zebrafish and grow these to adulthood. We then select adult fish carrying this genetic alteration in their sperm and eggs, and breed from those fish to generate a new generation (as many generations as is needed). During these breeding programs, we need to be able to identify fish carrying the genetic alteration, so we take a small number of cells (either from their fin which regenerates, or from their mucus on their skin), causing slight discomfort, and analyse the gene for the alteration. Once we have identified the genetically altered fish, we cross them to each other to make fish that have two genetically-altered copies of the gene, which we expect to show signs of the disease at some point during their life, which could cause pain, distress or harm. We then assess those potential disease models to see if they do actually show signs of the disease, and if so, we study them further up to the humane endpoint.

To understand the processes that change and their contribution to the disease (disease mechanisms), we sometimes need to manipulate other processes as well, either using chemicals that alter the function of proteins (pharmacological agents), by altering other genes, or by raising them differently to alter their body’s bacteria. We then examine the fish to see if this has made a difference. We can examine their movement or behaviour, or an invisible process happening within, which we visualise with a label such as a fluorescent tag, most often on fish that have been sacrificed for their tissue.

To search for treatments, we use embryonic and larval disease models to test large numbers of chemicals, and check to see if the signs of the disease are reduced by adding the chemical to the

water the fish are in. If we already have good reason to believe that a chemical might make a good treatment (e.g. data from cells with the disease), we might use adult zebrafish with the disease, but we will use embryos and larvae with the disease first if possible. In this case we might bathe the fish in the chemical or inject it. If our results are similar to those of our collaborators that have models of the same disease in other species or in cells, we can be more confident that the treatment will be beneficial for patients. The chemicals have potential to cause harm, so we use non-regulated embryonic and larval zebrafish (<5 days old) to determine the highest concentration that does not have a negative effect and then use that concentration on older zebrafish at regulated ages. We have some data suggesting that altering the body's bacteria alters the disease and seizure severity. We will generate zebrafish with a disease which have normal bacteria, or re treated with probiotics (beneficial bacteria) or prebiotics (food for beneficial bacteria). These manipulations all occur during non-regulated ages, but the fish may persist into regulated ages.

As a practical way to reduce the numbers of fish kept alive, and to preserve new strains, we take sperm from adult males and freeze it. We can then use this to re-derive the strain several years later using in vitro fertilisation (where eggs are taken from a female adult and mixed with the sperm to generate embryos that will grow into adult fish). This also reduces the effect of genetic drift on experimental results Genetic drift is where new genetic changes arise spontaneously and can affect the health of the zebrafish, which can change the results. Harvesting sperm and eggs causes mild discomfort.

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

Most adverse effects are expected to be transitory e.g. when taking mucus or fin samples there may be slight discomfort, which is treated with analgesia. Sometimes we will need to anaesthetise the animals eg when taking mucus or fin samples, performing electroretinography or electroencephalography (these are electrical recordings of the retina and brain function, respectively), or when imaging. When we do this, there is a small chance of an adverse reaction, in which case we will euthanise the fish straight away. It is possible that taking mucus or fin samples, massaging the belly to release eggs or sperm, or performing transplants could result in infection, in which case we will cull the fish as soon as this is noticed, or treat with antibiotics in the case of transplants. Massaging the belly to release eggs or sperm could result in damage to internal organs if not gentle enough, in which case we will cull the fish as soon as this is noticed.

The disease models are likely to show one or more of the following clinical signs: obviously small eyes; obvious weight loss; discolouration; abnormal swimming, behaviour, posture or balance; abnormal respiratory pattern (gill movements); unusual interactions such as aggression. We will determine a humane endpoint for each new disease model or mutant we generate.

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)?

About 50% of fish will experience mild severity and 50% will experience moderate severity.

What will happen to animals used in this project?

- Killed
- Used in other projects

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 diseases we work on predominantly affect the function of the central nervous system (eg learning and cognition) and retina (vision) but there is increasing evidence that they affect the peripheral nervous system (eg movement), and heart, and we know that the proteins function in all cells, so we expect symptoms to arise in other tissues if we manage to treat the brain and improve survival in the disease models. Hence, we need to test the effect of potential chemical treatments on the function of many organs.

I have used the Replacement Checklist at https://replacinganimalresearch.org.uk/resources/replacement-checklist/ and guidance at https://replacinganimalresearch.org.uk/resources/searching-for-alternatives/. I searched pubmed (a scientific literature database) and asked chatgpt (artificial intelligence) for models of CLN2, 3, 4 and 7 disease using ‘co-culture’, spheroid, retinoid, microphysiological, equivalent, biopsy, explant, in vitro, assay, organoid, organ-on a -chip, non-animal, 3D, microfluidic, in silico, machine learning, ex-vivo, in conjunction with: CLN, NCL, Batten and ceroid lipofuscin* in order to find non-animal (i.e. in vitro) models that exist for the diseases we study. I read the review papers and the more recent primary research papers to try to get the most recent information. I also asked ChatGPT, ‘is there an animal alternative or a combination of animal alternatives for testing the effect of a chemical for both efficacy and safety in all organs?' I also searched newsletters and social media from a variety of relevant charities for recent grants awarded for work in non-animal models. I also looked at clinicaltrial.gov and checked what disease models had been used in the past to develop new drugs for these diseases. This included looking at Patent applications.

ChatGPT suggested a combination of multiple organ-on-a-chip and human 3D cell cultures systems would be useful, some of which are available for some forms on the disease, but it also mentioned replacement animals such as fruit flies and nematodes, and partial replacement animals such as zebrafish embryos. So far, all chemicals identified as potentially therapeutic using cells modelling these diseases, followed by testing in one animal model, have failed in clinical trials except for Miglustat for CLN3 disease. Miglustat is still in clinical trial and looks promising. One reason for the success (so far) of Miglustat is that the pre-clinical tests in mouse and zebrafish animals studied symptoms and function rather than pathology or cellular processes. Many defects that can be assayed in vitro do not necessarily lead to symptoms and loss of function, with cell death and brain cell activity being exceptions e.g. seizures (excessive, co-ordinated brain activity) can be detected in brain organoids. Vision, coordinated motor function and autonomic regulation of the heart cannot yet be assayed in any in vitro culture system.

Hence, until additional in vitro models have been developed, a replacement animal model is a good alternative, especially as it gives safety and functional efficacy information at the same time. The fruit fly has the genes for CLN1, 3, 4, 7, 10, 12 and 13, but not for the other 6 genes that are involved in the diseases. For CLN3 disease, there is a fruit fly model, but it does not have any symptoms. For CLN4 disease there is a fruit fly with early death, but function has not yet been tested. At the moment, no fruit fly disease models are useful for testing chemicals to see if any chemical rescue function. The nematode has genes for CLN1, 3, 4, 7, 10, 11, 12 and 13, but not the other 5 genes involved in the diseases. Several models have early death and movement issues, but vision cannot be assayed. Our zebrafish models, most of which are, or are expected to be, partial replacement models as the experiments will be performed up to 5 days old, replicate the symptoms and function loss far better than fruit fly and nematode models. Zebrafish also have genes for CLN genes not found in fruit flies and nematodes e.g. CLN2 disease cannot be modelled in the fruit fly or nematode as the gene does not exist. We predominantly use zebrafish at the regulated age for mild procedures and to continue producing non-harmful genetic alterations in the next generation.

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

We employ replacement strategies where possible. These include collaborating to perform experiments in cell models (traditionally used mammalian cells, patient cells, human stem cells), human organoids and organs on a chip, the social amoeba, invertebrates such as the fruit fly, the nematode worm, and frog oocytes. We use unregulated embryonic and larval zebrafish when possible, depending on the availability of suitable models or whether the gene can be easily identified and mutated. We also take tissue from culled zebrafish and grow the cells in a dish.

Why were they not suitable?

These methods do not replicate the complex environment seen in a vertebrate organism, some models do not have the relevant genes, or the situation at later stages of development, but they do give us some information so we do these experiments to inform what experiments should be performed in zebrafish at protected ages.

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?

We expect to use up to 8000 of these fish for breeding purposes to supply embryos for experiments. This is based on 20 different strains (some are combinations of several strains, for which several intermediate strains need to be generated) with no harms caused by the genetic manipulation. Zebrafish are healthier when in groups of 20 or more (our calculation is based on an average of 40 fish

per tank), and new generations are needed about every 6 months - the old generations are not culled until the new generations are genotyped eg to find out whether or not they carry a specific genetic alteration. This is usually performed on adults. For some complex strains, only a small proportion of the fish are the correct genotype (carry the correct genetic alterations). This adds up to 8000 fish.

We expect to use 8000 (over a 5 year period) fish from genetically-altered strains at the regulated age for simple experiments where we document the harmful effects of the genetic alteration. Each experiment requires a pilot study (5-10 animals per group depending on the variability of the data from each assay, based on existing adult data or data from larvae) to calculate the number of animals required for a full study, and then each study should be replicated three times to ensure reproducibility. Assuming 75 animals (25 animals in each of three groups) per replicate, this allows for about 35 experiments over 5 years.

We expect to use 4000 (over a 5-year period) fish from genetically-altered strains at the regulated age for complex experiments where we document the potentially harmful effects of the genetic alteration in combination with a drug or chemical treatment, or alteration of the body's bacteria, for example. Each experiment requires a pilot study (see above) to calculate the number of animals required for a full study, and then each study should be replicated three times to ensure reproducibility. Assuming 150 animals (25 animals in each of six groups) per replicate, this allows for about 9 experiments over 5 years.

We will need to generate new strains of genetically-altered zebrafish and estimate 1000 animals will be needed. This is assuming 5% of animals will be able to transmit the new genetic alteration to their progeny and that we may make up to 50 strains.

In order to preserve newly created strains as frozen sperm, we expect to use 400 animals, which is approximately 10 animals for 40 new strains or combinations of strains.

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

We use the NC3Rs Experimental Design Assistant to design our experiments. This has led to us reducing bias by introducing randomisation (randomly selecting which animal goes into which group eg treatment or no-treatment) and blinding (not knowing what each group of animals were exposed to, or what genetic alternation they have, when you collect and analyse the results). Blinding is not always possible as some disease models are obvious when you look at them. For each experiment we also write 1) the objective, 2) a detailed description of the method and reagents needed (and ensure they are available) to minimise variance between researchers, 3) a calculation of the numbers of animals needed based on pilot studies, and 4) a description of the analysis method and the statistical test to be used.

We preserve sperm to reduce live animals, negating the need to recreate strains or combinations of strains. We use live animals for sperm and egg production to reduce animal numbers.

We are trialling genotyping of embryos and larvae using the Zebrafish Embryonic Genotyper, which gently removes cells from the skin for genotyping (determining the genetic alteration carried). Fish are not harmed and can be raised to adulthood without having had an invasive genotyping method.

Significantly, only fish with the required genetic alteration need to be raised beyond the regulated age (5 day old) to adulthood, reducing numbers.

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

Pilot experiments will be used to generate data for a power calculation to determine a suitable number of animals for a full replicate. For experiments using larvae, we usually find that 10 per group is acceptable unless the experiment is reporting locomotion or behaviour, which appears to be much more variable and sometimes requires 30 animals. Simple experiments comparing normal fish to a disease model tend to require less animals than an experiment where there is also a drug treatment, because the drug treatment may not completely rescue the signs of disease. In these cases, more animals are usually needed. For experiments new to us, we discuss animal numbers and data analysis with statisticians in advance. We repeat experiments up to three times to make sure the results are reproducible.

We aim to use the same animals for multiple assessments as long as we will not exceed the set severity limit.

We estimate the number of animals needed and breed what is required plus 20% to account for deaths during larval stages. Once zebrafish are genotyped, those not required for experiments or breeding are culled. We aim to genotype using the earliest, least harmful method possible.

We offer to share spare animals and tissue at our institution. They are often used for Schedule 1 training or as sentinels.

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 zebrafish as there is now a great deal of evidence that data from this vertebrate can be applied to humans due to the level of genetic similarity (known as conservation), similarity of relevant anatomy and highly similar responses to drugs. The models we have generated to date for CLN3 disease, CLN2 disease and East syndrome are no exception. In support of this conservation, we have evidence that several drugs first tested in cells works similarly in zebrafish to reduce CLN3 and CLN2 disease symptoms. One drug also treats the CLN3 disease mouse model, and another also treats the CLN7 mouse model. Tests in additional models are needed to show the drugs could treat more than one form of childhood neurodegeneration and epilepsy. The fast, external development of zebrafish means that many disease models can have clinical signs during unregulated embryonic and larval stages, and

these can be viewed easily without harming the mother. Where possible, unregulated larval zebrafish will be used.

The CLN2 disease model will be used before the regulated age of 5 days old. The CLN3 disease mutant model causes only mild symptoms (visual deficits and yellow colour) in adults. We do not yet know when the new disease models will display symptoms, but we aim to make models that replicate the genetic change in humans as best we can so that the model is as relevant to the disease as it can be.

The majority of animals will be maintained as non-harmful genetically altered families, minimising suffering. Animals that are maintained as harmful genetically altered families will predominantly be used for experiments that require us to observe the harmful consequences of the genetic alteration, though we aim to limit the harm caused as much as possible by choosing a suitable humane endpoint. Occasionally, we only have one genotyped animal for a particular strain. To avoid single housing, we will house them with animals of another genotype that have a visible difference such as fin length, pattern or coloration, if possible. If not possible, we will provide more enrichment.

Some of the animals that display harmful effects of the genetic manipulation will be further manipulated with genetic material, chemicals or drugs, by transplantation of tissue, or by altering their bacteria. Such experiments are required to show a drug treats the disease, or that a disease process really has negative consequences and should be targeted in treatments, which cells need to be corrected by gene therapy, or if altering the body’s bacteria impacts the disease. Transplantations will be performed during unregulated embryonic or larval stages to minimise harm and because the tissue integrates better into the host at this stage. Drug, chemical, or treatments affecting the body's bacteria will be first tested on unregulated embryos and larvae to determine the effective dose and frequency where possible, if there is no relevant information in the literature, or to confirm finding in the literature. The least invasive methods of delivery will be used where possible (bathing, in the food, oral gavage, then injection), depending on what is known about the metabolism of the agent, where it needs to be delivered to, and how it might be delivered in humans. In these experiments we also aim to limit harm by choosing a suitable humane endpoint. When we create new disease models and genetically-altered strains, only healthy fish are raised to the regulated age, to minimise harm. When we need to measure a clinical sign, we will design experiments to assess as many changes as possible (both live and under terminal anaesthesia) to gain maximum data from each animal.

Fish will be examined for the effects of their genetic alteration or treatment. The majority of these investigations will be observations of normal behaviour and locomotion, or tests on the tissue after euthanasia. We will perform some neurophysiological tests that require anaesthesia or restraint but are otherwise not harmful.

All these strains will need genotyping. We will use the least harmful method (fluorescent microscopy or deduction of genotypes from the offspring, both observed through microscopy during unregulated stages) and the earliest stage possible. When tissue samples are required, we will use embryo or larval skin cells (from the Zebrafish Embryonic Genotyper), or skin swabs, rather than fin clippings if the more refined method is reliable enough for identifying the specific genetic alteration. Skin swabs and fin clipping both use anaesthesia with recovery, but skin swabs are thought to be less harmful. Skin swabs can be performed without anaesthesia, but it is not yet clear how the stress induced because the fish are awake compares to stress from anaesthesia. We have recently begun using skin

swabs and will need to show that the results using this method are as good as with fin clips for each strain before we can use skin swabs exclusively.

We will need to preserve sperm, which allows us to freeze these strains and maintain less fish alive. These strains can later be resurrected using in vitro fertilisation. Anaesthesia is used when taking sperm or eggs. Fish recover and can be used to provide sperm or eggs once more, meaning less fish are required. Currently, expert collaborators preserve sperm and perform in vitro fertilisation for us. We aim for them to train us in this method if we bring it in house.

Whenever anaesthesia is used, the required dose is carefully tested for the first fish (ensuring a quick and healthy recovery after the procedure) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish.

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

Where possible, we will use animals at more immature life stages which are less sentient. This will depend on the stage when the genetic manipulation causes changes that are measurably different from wild type zebrafish. When we do not need to measure a clinical sign, we will measure changes using non-harmful methods such as videoing their locomotion, plus changes that be observed in tissue from animals culled or terminally anaesthetised prior to the age when the clinical sign is evident.

We favour the use of zebrafish over invertebrates as the zebrafish brain and retina is considerably more similar to the mammalian brain and retina. As zebrafish are the genetic vertebrate model which respond least to stimuli, they are also our preferred vertebrate for experiments at regulated stages. We also feel that the zebrafish is the most refined model for the research questions we are addressing.

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

We have a welfare monitoring sheet to know what signs of harm or ill-health to look for (affecting locomotion, posture, tank position, skin and fin condition, gill movement and operculum condition, body shape) and what to do when we find fish with those issues, which can include increased monitoring or culling within a certain timeframe if no improvement is seen eg at the last welfare check of the day to avoid a long un-monitored period such as overnight. The form has space for notes and for sharing plans.

We are trialling genotyping embryos at less than 5 days old using the Zebrafish Embryonic Genotyper, which gently removes cells from the skin for genotyping. Fish are not harmed and can be raised to adulthood without having had an invasive genotyping method.

We are also trialling genotyping in adults using swabbing, which is potentially less invasive than removing some of the fin.

The dose of anaesthesia is carefully checked for the first fish (ensuring a quick and healthy recovery after the procedure) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish.

Analgesia is used prior to, during and after fin clipping.

Occasionally, we only have one genotyped animal for a particular strain. To avoid single housing, we will house them with animals of another genotype that have a visible difference such as fin length, pattern or coloration. If we do not have any suitable fish to house them with, and need to keep fish in a tank on their own, we will provide plants and other environmental enrichment to reduce stress and only house them alone for a maximum of 72 hours.

Transplantations will be performed during unregulated embryonic or larval stages to minimise harm and because the tissue integrates better into the host at this stage.

Drug, chemical or gene therapy treatments will be first tested on unregulated embryos and larvae to determine the effective dose and frequency where possible if there is no relevant information in the literature. The least invasive methods of delivery will be used where possible (bathing, in the food, oral gavage, then injection), depending on what is known about the metabolism of the agent, where it needs to be delivered to, and how it might be delivered in humans.

We will create new disease models and genetically-altered strains. Only healthy fish are raised to the regulated age, to minimise harm.

Once we have created a new genetically-altered strain, we will develop a score sheet for that strain so that expected phenotypes are known and specifically looked for. This will be based on the existing score sheet but contain additional elements based on the expected phenotypes eg seizures, difficulty swimming, and difficulty finding food. Routine locomotion assays will enable us to identify subtle phenotypes that are not noticeable with the naked eye. For fish with visual deficits, they may have difficulty locating food, in which case we will provide food that remains suspended in the water for longer. With regards to humane end-points, fish with abnormal but stable phenotypes such as abnormal locomotion or posture would be monitored and kept for a maximum of seven days. Seven days should give us sufficient time to perform non-harmful assays such as locomotion assays. On the other hand, fish would be culled within one day if unable to feed or their health was deteriorating fast. In both cases, case we might perform some assays under anaesthesia and then immediately cull them. For intermittent phenotypes such as seizures, monitoring will be increased to ascertain the frequency so we can determine the level of harm and decide on a humane end-point (up to seven days).

The protocols state the minimum required monitoring provided. Monitoring will be increased if any fish respond adversely to the procedure.

When studying adverse effects, we aim to limit the harm caused as much as possible by choosing a suitable humane endpoint.

We do not keep animals beyond 18 months old and frequently cull them earlier if the next generation is genotyped and has reached adulthood.

We will use Schedule 1 methods for euthanasia, rather than another humane killing method, whenever it does not matter for the study.

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

We will follow Home Office guidance and the most refined published methods and discuss them with the Named Animal Care and Welfare Officer and the Named Veterinary Surgeon.

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

We seek advice from our NVS and large zebrafish facilities such as UCL and keep abreast of latest refinements via the British Association of Animal Husbandry, the European Zebrafish Society and their meetings, the Lab Animal Science Association, the NC3Rs, the Institute for Animal Technology and the journal Zebrafish. We receive newsletters and bulletins from these groups. We search the literature for more refined methods. I was a committee member for the European Zebrafish Society when they reviewed use of anaesthetic.

We have meetings every few months with other research groups using fish and the staff that care for the fish, where we present our research so they can feed back on any best practice they are aware of.

We will keep up to date with changes in the ARRIVE guidelines for the reporting of animal experimentation.

When we find a new method we believe to be best practice, we contact the developer and arrange for training.