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Multi-allelic phenotyping – A systematic approach for the simultaneous analysis of multiple induced mutations ☆

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ABSTRACT

The zebrafish mutation project (ZMP) aims to generate a loss of function allele for every protein-coding gene, but importantly to also characterise the phenotypes of these alleles during the first five days of development. Such a large-scale screen requires a systematic approach both to identifying phenotypes, and also to linking those phenotypes to specific mutations. This phenotyping pipeline simultaneously assesses the consequences of multiple alleles in a two-step process. First, mutations that do not produce a visible phenotype during the first five days of development are identified, while a second round of phenotyping focuses on detailed analysis of those alleles that are suspected to cause a phenotype. Allele-specific PCR single nucleotide polymorphism (SNP) assays are used to genotype F2 parents and individual F3 fry for mutations known to be present in the F1 founder. With this method specific phenotypes can be linked to induced mutations. In addition a method is described for cryopreserving sperm samples of mutagenised males and their subsequent use for *in vitro* fertilisation to generate F2 families for phenotyping. Ultimately this approach will lead to the functional annotation of the zebrafish genome, which will deepen our understanding of gene function in development and disease.

1. Introduction

Whole genome sequence is now available for humans as well as a number of other vertebrate species. While this has provided detailed information about the position and sequence of protein coding genes, the functions of these genes and their roles in development and disease remain largely unknown. Loss of function analysis can be either an appealing starting point or an integral component in the endeavour to elucidate gene function.

Forward genetic screens [1–6] have typically taken advantage of mutagenic chemicals such as EMS (Ethyl methanesulfonate) and ENU (*N*-ethyl-*N*-nitrosourea) to produce rich collections of phenotypes. Identification of the underlying mutations responsible for these phenotypes still poses significant challenges even today [7].

The completed sequence of the zebrafish (*Danio rerio*) genome and its detailed annotation has yielded the sequence of more than 26,000 zebrafish protein coding genes [8,9]. Previously we have shown that it is possible to amplify specific exons across a large library of ENU mutagenised individuals and sequence these amplicons in order to identify mutations of interest, but these approaches are limited by the need for PCR amplification [10]. Alternatively, specific genomic regions can be enriched by hybridising fragmented DNA to selected regions of interest [11]. This has been used successfully to enrich genomic DNA sequencing libraries for whole exome analyses in human [12] and mouse [13]. To apply this technique in zebrafish, Agilent SureSelect™ RNA baits were designed to enrich for all protein coding exons identified in the Zv8 and Zv9 zebrafish genome assemblies [14]. Combined with Illumina sequencing and single nucleotide variant (SNV) calling [15–17], this method reliably identifies point mutations in thousands of individual fish thus taking advantage of the high mutagenic load ENU creates [18] (Fig. 1b). Sperm from the sequenced F1 males (Fig. 1a) is archived by cryopreservation and therefore desired alleles can be prioritised for *in vitro* fertilisation and revived into F2 families (Fig. 1b). Competitive allele-specific PCR SNP genotyping assays (KASP™, KBioscience) are designed [19] for the identified mutations and published on the ZMP website (Fig. 1b). The phenotypic consequences of all non-synonymous alleles contained in the preserved founder are addressed in a multi-allelic phenotyping pipeline, creating a crossroads of both forward and reverse genetics [20].

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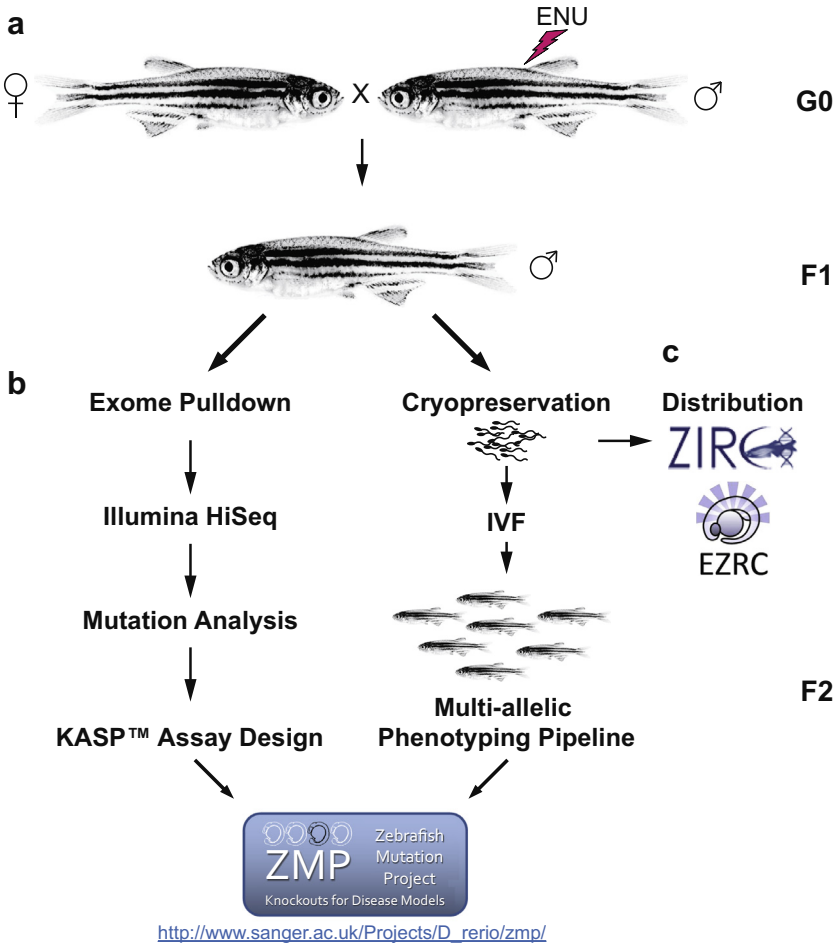

Fig. 1. Mutation detection overview. Male TLF zebrafish (G0) are treated with ENU and then out-crossed (a) to produce F1 families heterozygously carrying induced mutations. F1 fish (b) are raised to an age of about 12 months and sperm is collected from the fish. Each individual is then sacrificed and tissue samples of the body are taken. The tails are used for genomic DNA preparation while the rest of the body is preserved for archival purposes. Genomic DNA is then subjected to exome pulldown (b), sequenced via Illumina paired end HiSeq and analysed to detect induced mutations. KASP™ assays are designed for each allele and then all information is made available on the ZMP website as the project proceeds. The cryopreserved sperm is made available to ZIRC and EZRC where alleles can be ordered. (c) An aliquot of sperm from each F1 male is retained at the Wellcome Trust Sanger Institute and used for the IVF of F2 families which are placed into the ZMP multi-allelic phenotyping pipeline. Phenotypic descriptions are published on the ZMP website.

2. Cryopreservation

Cryopreserving sperm of F1 individuals into multiple aliquots as they are being sequenced allows for permanent archiving of identified alleles, distribution and selection of families to phenotype based on allele composition (Fig. 1c). Several zebrafish sperm cryopreservation protocols have been described previously [21–23]. They are based on either dissection of testis, thereby sacrificing the males, or retrieval of sperm by abdominal massage and use different types of cryoprotectants yielding different numbers of samples per male. The method described here is a combination and modification of protocols mentioned above. It uses N,N-dimethylacetamide (DMA) in buffered sperm-motility inhibiting solution (BSMIS) as the cryoprotectant and yields eight samples per male. As males are not sacrificed during this procedure they can be reused after a rest period.

Males that are to be used for cryopreservation should be between 6 and 12 months old. It is important to keep them at a low stock density and to feed them well to generate relatively large fish. Sperm quantity can be increased significantly by separating males from females at least a week in advance of cryopreservation. Sperm quantity and quality do not deteriorate by prolonged sex separation. A well-fed male that has been separated from females can easily produce 4 μl of high density sperm.

Depending on the number of samples to be frozen, ideally two or three people should work together on this protocol. One person opens cryovials (Corning Product #430659), retrieves the sperm and aliquots it into the cryovials while a second person anaesthetises males, records data and closes and transfers cryovials into the 50 ml Falcon tubes on dry ice and later into liquid nitrogen (LN_2). For maximal throughput a third person can take over opening and closing of Falcon tubes and moving of cryovials into LN_2, while the second person focuses on fish anaesthesia, data records and transfer of vials into Falcon tubes. If tissue samples for DNA isolation are also desired, a fourth person sacrifices the squeezed male fish by over-anaesthesia, takes tissue samples such as the tail fin and a section of the trunk and places those into a 96 deep well block on dry ice. It is imperative that the location of the tissue sample in the deep well block and the corresponding sperm sample are properly documented. Working equipment should be prepared 30 min in advance to allow for tubes to cool down. A workstation set up for cryopreservation is depicted in Fig. 2a.

Firstly a solution of 10% DMA in BSMIS (75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris pH 8.0, sterile filtered