Reverse genetics in zebrafish by TILLING

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Advance Access publication date 21 November 2008

Abstract
TILLING, for Targeting Induced Local Lesions in Genomes, is a reverse genetics strategy that identifies mutations in specific genes of interest in chemically mutagenized populations. First described in 2000 for mutation detection in Arabidopsis, TILLING is now used in a wide range of plants including soybean, rice, barley and maize as well as for animal model systems, including Arabidopsis, Drosophila, Caenorhabditis elegans, rat, medaka and zebrafish and for the discovery of naturally occurring polymorphisms in humans. This review summarizes current TILLING methodologies as they have been applied to the zebrafish, ongoing TILLING projects and resources in the zebrafish community, and the future of zebrafish TILLING.

Keywords: zebrafish; TILLING; Cell mismatch cleavage; resequencing; reverse genetics

HISTORY OF TILLING
Mutagenized populations carry a large number of potentially deleterious recessive mutations that can be discovered based on the phenotypes of homozygous mutants (forward genetics) or by detecting them directly in the genomic DNA of heterozygous or homozygous individuals, irrespective of any phenotypes they may cause (reverse genetics). In the latter case, the challenge is to identify rare mutations within a large population of non-mutant individuals. This process, called ‘TILLING’ has been applied to a range of plant and animal species [1–11]. The idea of screening mutagenized populations of zebrafish by the polymerase chain reaction (PCR) for mutations in genes of interest was first proposed for mutagens that cause deletions [12, 13]; however, the challenges of working with large deficiencies limited this approach. A key advance was made by McCallum et al. [14], who used automated denaturing high-performance liquid chromatography to detect heteroduplexes resulting from rare single nucleotide polymorphisms (SNPs) in pooled ethyl methanesulfonate (EMS)-mutagenized Arabidopsis populations. They coined the term ‘TILLING’ to describe this general approach. Soon after, the same group introduced the use of the Cel1 enzyme isolated from bulk celery to detect polymorphisms in these populations [15]. Cel1 is a plant-specific extracellular glycoprotein that cleaves heteroduplex DNA at all possible single nucleotide mismatches [16]. Using fluorescently labelled primers to detect Cel1 cleavage products on a LiCor acrylamide slab gel, Colbert et al. [15] showed that Cel1 could detect a heterozygous SNP in pools of eight mutagenized plants. This pooling strategy allowed a 1 kb fragment to be screened in a library of 3000 mutagenized plants in three 96-well LiCor runs—a throughput of one gene per day.

The Cel1 method of mutation detection was applied to zebrafish at the Netherlands Institute for Developmental Biology at the Hubrecht lab and in our group in Seattle [8, 17]. At the Hubrecht lab, the Cel1 approach was quickly superseded by the alternative approach of direct resequencing of target genes from single mutagenized fish [18–20]. The phenotypes of a number of mutants generated by TILLING (through Cel1 or resequencing) have been published [19–27]; however, ongoing TILLING projects have generated loss-of-function mutations that are yet to be published in over 150 zebrafish genes (see http://www.sanger.ac.uk/Projects/D_rerio/mutres/ and http://www.zfishtilling.org/zfish/) and which are expected to provide insights...
into mechanisms of development and disease in this vertebrate model system.

**TILLING METHODOLOGY APPLIED TO ZEBRAFISH**

The general approach for TILLING in zebrafish is simple: genomic DNA from a large library of ENU-mutagenized zebrafish is screened for rare mutations in genes of interest either using Cel1 on pools of fish or by resequencing individual fish. Mutations are then recovered by out-crossing the single identified carrier. The key components of a successful TILLING project are (i) a large, well-mutagenized library of fish, (ii) an efficient screening method and (iii) a near-perfect ability to recover valuable mutations once they have been found.

**Libraries**

For any TILLING approach (Cel1 or resequencing), a large library of ENU-mutagenized individuals, whose genomic DNA can be screened for mutations in genes of interest, is essential. The ENU-mutagenesis regimen for zebrafish TILLING is the same as that used for forward genetic screens: four to six weekly 1-hr treatments with 3–3.5 mM ENU. Since TILLING can identify mutations in the heterozygous condition, it is possible to screen the F1 offspring of a zebrafish mutagenesis, each of which is heterozygous for a large number of unique ENU-induced mutations. TILLING libraries can either be ‘living’ or cryopreserved. A living library is one in which mutagenized fish (male and female) are held in pools in tanks while their fin-clip genomic DNA is screened; in this scenario mutants are recovered by out-crossing the identified F1 fish. A cryopreserved library is one in which each genomic DNA sample corresponds to one or more vials of frozen sperm that can be used in an in vitro fertilization to generate F2 heterozygotes. As a cryopreserved library is stable for many years (we have observed no significant loss of fertility of our library in over 6 years; Figure 1), and since a large amount of genomic DNA can be prepared from the carcass of a single adult male fish, the cryopreserved sperm and corresponding genomic DNA is a long-term TILLING resource.

**Choice of TILLING targets**

A number of factors determine what part of a zebrafish gene of interest makes the best TILLING target. The upper size of fragments that can be screened with Cel1 is 1.5 kb, less for resequencing. Since it involves the same time and expense to screen small exons as to screen large ones, and since the chances of finding a deleterious mutation is expected to scale with the number of base pairs screened, large exons are highly prized. Screening 5’-exons is preferable if the goal is to find null or strongly hypomorphic alleles, since nonsense mutations early in the coding sequence are most likely to result in a non-functional truncated protein. However, since large exons are the exception rather than the rule for zebrafish genes, frequently it is necessary to screen multiple exons to find a loss-of-function mutation. When large exons are available, web-based tools such as CODDLE (http://www.proweb.org/coddle/) can help to identify the region of the exon that has the highest likelihood of containing nonsense mutations, given the mutagenic spectrum of ENU in zebrafish, and to identify regions encoding conserved domains where missense mutations are likely to be deleterious.

**Cel1 screening**

Detailed protocols for Cel1 preparation and screening of zebrafish DNA are available online (http://labs.fhcrc.org/moens/protocols). The methodology for zebrafish is essentially as described by Colbert.
et al. [15] for Arabidopsis with some modifications. In order to avoid over-representation of particular genomes in pooled samples, genomic DNA from F1 fish must be normalized before pooling. Cel1 digestion can detect mutations that are represented as only 1/16 alleles, so genomic DNAs of heterozygous fish can be pooled up to 8-fold and arrayed in 96-well 'pool plates’. Genomic DNA is amplified from pooled DNA using opposed PCR primers carrying different dye labels [infrared (IR) dye 700 and IR dye 800], and PCR products are denatured and allowed to re-anneal to generate heteroduplexes with mismatches at the sites of both ENU-induced mutations and pre-existing polymorphisms. Re-annealed products are then incubated with a Cel1 concentration sufficient for partial digestion, so that induced mutations can be detected in the background of the pre-existing polymorphisms that are present in ~1/600–1/300 nt of coding DNA in standard zebrafish strains [28]. Digested products are separated by acrylamide electrophoresis using a LiCor DNA analyser and the resulting images are analysed using 'GelBuddy' software to [29] (http://www.proweb.org/gelbuddy/). Since Gelbuddy mark-ups are recorded as tab-delineated text, gel data can be uploaded directly into a database that tracks mutations from their initial identification in pooled DNAs through Cel1 re-identification from the individuals that constitute the pool, to their ultimate validation by sequencing (e.g. http://www.zfistilling.org/zfish/).

Analysis of almost 1000 zebrafish mutations detected with Cel1 in our lab confirms that this approach effectively detects all classes of ENU-induced mutations and pre-existing polymorphisms. Mutations identified by TILLING (black bars) and in forward genetic screens (grey bars) are grouped into one of six possible classes of mutation. TILLING data are based on 982 mutations identified in the Moens lab. Forward screen data were mined from the primary literature for an arbitrary subset of zebrafish mutations that were identified through positional cloning.

**Figure 2:** Similar spectra of ENU-induced mutations from forward and reverse genetic approaches in zebrafish. Mutations identified by TILLING (black bars) and in forward genetic screens (grey bars) are grouped into one of six possible classes of mutation. TILLING data are based on 982 mutations identified in the Moens lab. Forward screen data were mined from the primary literature for an arbitrary subset of zebrafish mutations that were identified through positional cloning.

**Resequencing**

Mutation identification by direct resequencing of ENU-mutagenized F1 zebrafish was first described for the zebrafish rag-1 gene [20]. The general approach involves using nested PCR to amplify and sequence gene fragments of interest from a large library of single fish, followed by sequence alignment and heterozygosity detection with base-calling programs, such as polyPhred [30]. The approach requires significant liquid-handling capacity to perform multiple consecutive reactions on thousands of individual fish. As such, it is most effectively performed at highly roboticized sequencing centres, such as the Sanger Center. A productive collaboration between the Netherlands Institute for Developmental Biology at the Hubrecht Lab and the Sanger Center, funded by the European ZF-Models program, has generated deleterious mutations in over 100 zebrafish genes by resequencing genomic DNA from living libraries. Users can request genes to be screened online at http://www.sanger.ac.uk/Projects/D_rerio/mutres/.

**Mutant recovery**

Mutations are recovered either from living F1 fish or from their cryopreserved sperm by out-crossing to generate heterozygous F2 progeny. Mutations can be re-identified in F2 fin-clip DNA by direct sequencing or by detection of a restriction length polymorphism (RFLP) created by the mutation. When no RFLP is available, it is possible to design one into nearly matched allele-specific genotyping primers (‘dCAPS’ primers; http://helix.wustl.edu/dcaps/dcaps.html) [31]. Identified F2 heterozygotes can be intercrossed or, preferably, crossed to fish heterozygous for another allele of the same gene, to evaluate gene function.

**PROBLEMS WITH TILLING**

The single major drawback of TILLING as a method for reverse genetics is that a mutation of interest that is identified in any given F1 fish is only one of
many heterozygous mutations in its genome. By multiplying the estimated size of the zebrafish genome (1.45 × 10^3 Mb) by the mutation rate detected by TILLING (2–4 mutations per mega-base), an estimate of the number of ENU-induced mutations per F1 fish is ~3–6000. As a result, a mutant phenotype observed in a clutch of eggs from a pair of F2 fish both heterozygous for a mutation identified by TILLING is likely to be due to another ENU-induced mutation(s). Since TILLING mutants are generated by the same mutagenesis regime used for forward genetic screens, the criteria for attributing a phenotype to a mutation identified by TILLING should ultimately be the same as for attributing a mutant phenotype to a mutation identified through a positional cloning approach: minimally, co-segregation of the phenotype and the mutant genotype in a large number of fish; preferably, phenotypic rescue with the wild-type gene; and ideally, a second non-complementing allele from a different F1 male. When TILLING identifies two deleterious alleles in a single gene, both alleles can be recovered so that the mutant phenotype can be studied unambiguously in compound heterozygotes. Where genomic deletions in the region of interest are available, the TILLING mutant can be placed over this deficiency. Indeed, an unanticipated use of TILLING has been to generate non-complementing alleles in genes identified by positional cloning for which only one allele was originally identified in a forward genetic screen.

We have noted that the number of mutations identified by TILLING does not necessarily correspond with the size of the fragment being screened (Figure 3). This suggests that our ability to detect mutations depends on the fragment being screened, a technical problem with the Cel1 screening method, in particular. Low mutation frequencies tend to correlate with the presence of multiple pre-existing polymorphisms in the fragment, since the presence of polymorphisms reduces the number of Cel1 cleavage products that are both cut at the induced mutation and retain the labeled primers. In these cases it is necessary to screen other regions of the same gene, or to use an alternative screening strategy such as resequencing.

THE FUTURE OF TILLING

TILLING is the most established method of reverse genetics available in the zebrafish; however, new methodologies, reviewed in this volume, are becoming available. Insertional mutagenesis projects using pseudotyped retroviruses have produced large libraries of F1 fish carrying as many as 25 random heterozygous insertions that can be sequenced and mapped to produce an indexed library of mutant loci recoverable from cryopreserved sperm [32] (see chapter by S. Burgess). Because retroviruses tend to integrate into the first introns of genes or into exons, many of these integrations are mutagenic as determined by a strong reduction in RNA levels.

![Figure 3: The number of mutations found in a fragment by TILLING with Cel1 corresponds poorly with fragment size. Thirty-six genes screened using Cel1 in the Moens lab are ranked by the size of the exon(s) that were screened (grey bars, in base pairs × 10^2). Black bars represent the number of mutations that were identified in those exons.](image-url)
Zinc-finger nucleases, which can be designed to deliver frameshift mutations at defined target sites in the genome, have also recently been applied to zebrafish [33, 34] (see chapter by S. Amacher). To date, TILLING efforts have focused on identifying loss-of-function mutations, in the form of nonsense or splice alleles; however, as these other methods for generating loss-of-function mutations become more accessible and affordable, the unique ability of TILLING to generate allelic series, including a range of hypomorphic and even temperature-sensitive alleles caused by missense mutations, will become increasingly valuable. To this end, we and others continue to explore ways to improve the effectiveness and efficiency of TILLING methodologies.

Although current TILLING projects have produced loss-of-function alleles in over 150 zebrafish genes to date, the general approach is limited by the size of the available libraries and the low-to-medium throughput of the screening methodologies. To address the first of these, we have established a consortium of three labs (C. Moens, L. Solnica-Krezel and J. Postlethwait), who have independently established cryopreserved TILLING libraries and Cell screening methodology. Consortium-based screening will improve the likelihood of finding one or more loss-of-function alleles of genes submitted by the zebrafish community. Currently, the largest cryopreserved TILLING library of 8640 F1 fish has only a 58% chance of containing a nonsense mutation in any given 1 kb of coding sequence. This number climbs to 83% in the combined libraries of the consortium labs. By late 2008, members of the community will be able to submit requests to the consortium online. The zebrafish community will also continue to have access to the resequencing power of the Sanger Center with the ongoing Zebrafish Mutation Resource offered by the Stemple lab (http://www.sanger.ac.uk/Projects/D_rerio/mutres/).

New screening methodologies are needed to address the issue of throughput. In our hands, Cell screening proceeds at a rate of about one fragment per week. Automated resequencing projects, such as the Sanger Center’s, have a significantly higher throughput but remain labour and machine intensive. The advent of massively parallel sequencing platforms, that can generate more than half a billion base pairs of high-quality sequence per run, can be applied to TILLING. Here, the challenge is to uniquely identify genomic DNA templates from individual fish in large pooled populations, and to enrich pooled genomic DNAs for targets of interest to reduce the complexity of the sequencing template. By ligating oligonucleotide ‘barcodes’ to sheared genomic DNA from individuals or small pools, it may be possible to screen ‘superpools’ representing up to 8000 individuals in a single Solexa or equivalent sequencing run. Template DNA can be enriched for targets of interest by gene-specific PCR or by hybridization capture to custom microarrays [35] or streptavidin beads loaded with biotylated target-specific oligonucleotides. Using these tools it may be possible to screen as many as 50 500 bp targets regions in a single 8-lane Solexa run—a significant improvement in throughput and cost over either current methodology.

**Key Points**

- TILLING is a method for identifying induced mutations in chemically mutagenized genomes.
- Zebrafish TILLING projects have identified over 150 loss-of-function mutations in genes that have not been identified in forward genetic screens.
- TILLING can be performed by mismatch detection using the enzyme Cel1 on pools of mutagenized F1 fish, or by direct resequencing of individual fish.
- TILLING is complementary to other reverse genetics approaches that are being developed in zebrafish.
- New approaches that will improve the effectiveness and efficiency of TILLING in zebrafish are being developed.

**References**


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