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# Lessons from morpholino-based screening in zebrafish

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## Abstract

Morpholino oligonucleotides (MOs) are an effective, gene-specific antisense knockdown technology used in many model systems. Here we describe the application of MOs in zebrafish (*Danio rerio*) for *in vivo* functional characterization of gene activity. We summarize our screening experience beginning with gene target selection. We then discuss screening parameter considerations and data and database management. Finally, we emphasize the importance of off-target effect management and thorough downstream phenotypic validation. We discuss current morpholino limitations, including reduced stability when stored in aqueous solution. Advances in MO technology now provide a measure of spatiotemporal control over MO activity, presenting the opportunity for incorporating more finely tuned analyses into MO-based screening. Therefore, with careful management, MOs remain a valuable tool for discovery screening as well as individual gene knockdown analysis.

***Keywords:*** *morpholinos; zebrafish; knockdown*

## MORPHOLINO-BASED SCREENING IN ZEBRAFISH

Knockdown screens searching for novel genes and pathways have been conducted using a variety of technologies and model organisms. Early examples include [1] in the nematode and [2] in fly tissue culture cells. Successful screening is synergistic, combining the respective advantages of a specific model organism and technology to reveal unknown biology. Nevertheless, each protocol has inherent biases based on the selected screening parameters and knockdown tool efficacy and specificity. Here we summarize screening with zebrafish (*Danio rerio*) as a model organism using antisense morpholino oligonucleotides (MOs) as the primary gene-specific knockdown approach.

Zebrafish embryos are transparent and develop externally, a combination of properties which makes this an outstanding model organism for studying early vertebrate development. Embryogenesis is complete by 50 hours postfertilization (hpf) with the development of nearly all major vertebrate organ systems firmly established. MOs were our knockdown tool of choice because of the ease of delivery and their high efficacy throughout zebrafish embryonic and larval development (for a comprehensive review of MO use and zebrafish embryo injection technique see, [3, 4]). MOs are a synthetic derivative of DNA with two major changes: (i) a six-membered morpholine ring replaces the standard deoxyribose ring, and (ii) a non-ionic phosphorodiamidate linkage replaces the anionic phosphodiester bond [5]. The resulting neutral charge and relatively small size (usually 25 bases in length) make diffusion the main driving force of spread throughout the embryo following microinjection [6]. MOs are extremely effective through 50 hpf but can lose efficacy in a sequence-specific manner thereafter (see [4] for review). Although we and others largely focused on this traditional efficacy window, morpholino-based screening has also been effective at identifying novel loci critical for later developmental processes (e.g. [7]).

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**Victoria M. Bedell** is an MD/PhD student using morpholinos for in-depth study of one of the genes found during this screen.
**Stephanie E. Westcot** is a JD/PhD student who has used morpholinos to study skin development in zebrafish.
**Stephen C. Ekker** has a long-standing interest in zebrafish biology and developed the first use of morpholinos as gene-specific knockdown agents for this vertebrate.

Conventional forward genetic approaches are well established for the zebrafish [8–10]. Although these methods have generated numerous mutant alleles, large-scale genetic analysis in vertebrates is costly and both time- and labor-intensive including the breeding, maintainance and analysis of many fish over multiple generations. Classical chemical mutagenesis screens efficiently generate altered genetic loci but molecular characterization of the altered loci remains relatively slow and arduous. Insertional mutagens often permit accelerated locus identification, but they still require housing and breeding of many normal fish to generate the subset of mutant animals for analysis. Therefore, a key advantage of MO-based screening is the ability to generate knockdown phenotypes in wild-type embryos, significantly reducing infrastructural needs as compared to a classical mutagenesis screening.

Furthermore, phenotypes obtained from random mutagenesis approaches may be due to a gene whose function has already been well characterized in previous work, resulting in potential redundancy of efforts as our knowledge base of core vertebrate genes grows. Because MOs must be designed against specific sequences, an additional advantage of MO-based screening is that it readily integrates bioinformatics information into selecting target genes of interest, thus minimizing redundancy during the screening effort.

## SCREENING DESIGN: BIOINFORMATICS AND TARGET GENE SELECTION CRITERIA

Target gene identification is the first step in MO screening. We selected the secretome—a subset of the genome encoding ligands, receptors and extracellular molecules—because cellular context and cell–cell signaling play extensive roles in vertebrate biology and physiology [11, 12]. In particular, we used bioinformatics tools to compare the transcriptomes of the human, pufferfish (*Fugu rubipes*) and zebrafish genomes to identify putative co-translationally translocated (CTT) protein sequences [11, 13]. CTT secretome members have hallmark amino-terminal signal sequences that can be readily identified using secondary structure prediction tools [14]. The combination of signal sequence prediction and comparative genomics analysis establishes likely translational start sites for members of the zebrafish secretome [13, 15] (Figure 1A). Start site identification is a critical step in MO design for large-scale morpholino-based gene targeting because MOs are extremely effective as translation inhibitors when targeted to the mRNA start codon or upstream (non coding) sequences [3–6]. For detailed, comprehensive discussions of the advantages and disadvantages of translation-blocking versus splice-site targeting MOs, see [3, 4].

## SCREENING PROCESS: INJECTING THE MO AND SELECTING SCREENING DOSE

Standardization of the injection process, development of a reproducible dose response curve, rapid phenotyping, detailed data description and central data collection were all critical elements for effective morpholino-based screening. We will discuss each of these topics in further detail.

Two elements are key to standardizing MO injection: (i) the fishes' genetic background, and (ii) the MO dose. First, a parent population that reliably provides large clutches of uniformly sized embryos with little death or deformity greatly facilitates the screening process. The object is to reduce the amount of genetic background 'noise' so as not to obscure the downstream phenotypic 'signal'.

Second, standardizing MO dosing requires determining the dose range within which most MOs will elicit a specific phenotype without overt non-specific effects. We chose to define a 95% confidence interval wherein most MOs would be >50% penetrant (i.e. >50% of injected embryos showed a specific phenotype). To standardize the doses, we performed a prescreen calibration using 20 MOs targeting genes with known embryonic loss-of-function phenotypes [16]. Nineteen of these initial 20 MOs yielded >50% penetrance between 1.5 and 6 ng. Indeed, most approached 90% penetrance within that dose range (Figure 1B).

Using a dose curve rather than a single dose for each MO also provides information regarding phenotypic severity and MO toxicity. As MO dosage increases, phenotypic penetrance improves, which in turn increases phenotypic severity [6]. This remarkable effect allows us to examine particular components of a phenotype by adjusting MO dosage. However, with increasing MO dosage comes greater MO toxicity (as discussed below; [17]). The dose curve also provides information on

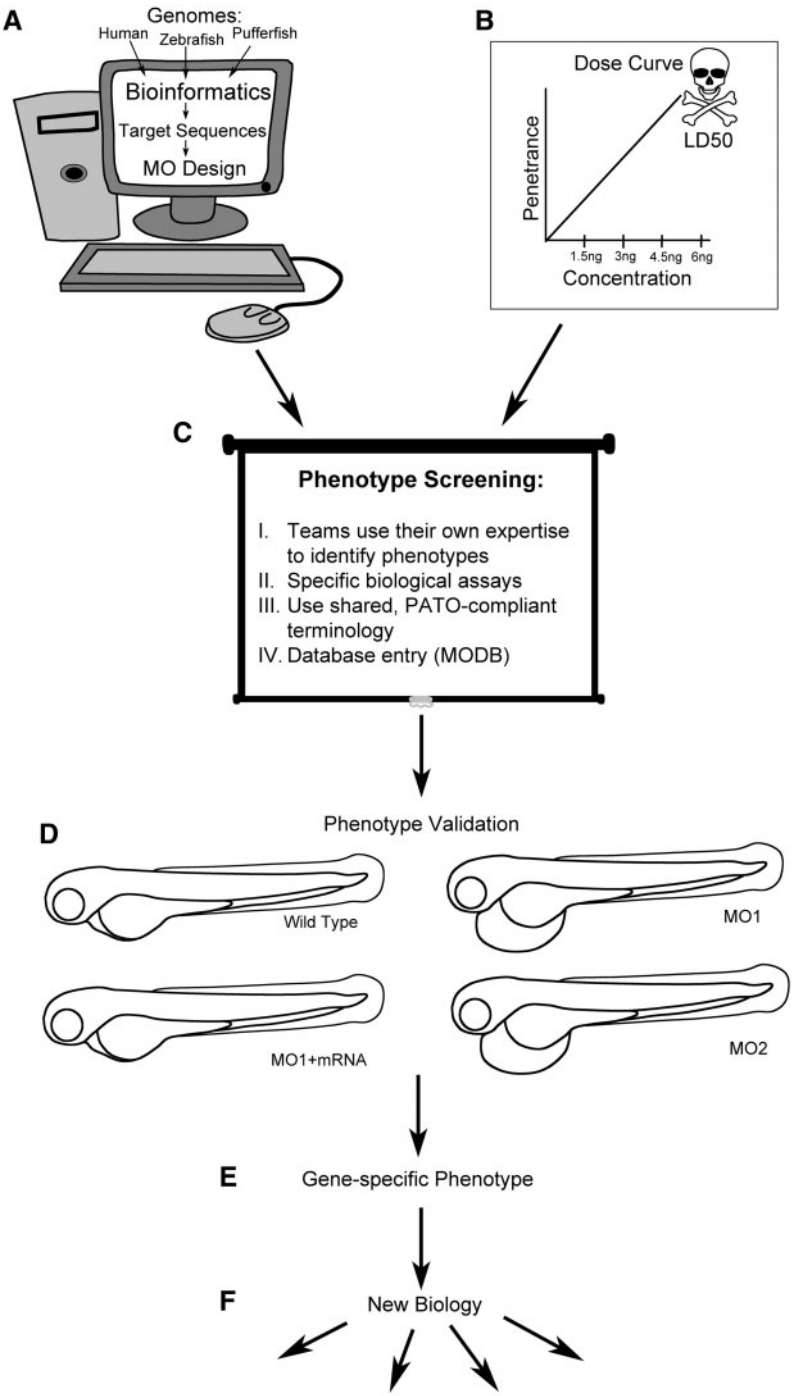


**Figure 1:** Workflow using morpholinos as a genetic screening tool. (**A**) The first step is to identify the target genes to be screened. We identified our target sequences using bioinformatics tools and comparing human, zebrafish and pufferfish genomes. Once the likely start site was identified, the MO was designed using standard MO-mRNA target sequence binding parameters (reviewed in [4]). (**B**) Second, we established a standard dose curve. We designed 20 MOs against genes with known phenotypes to establish a broad calibration curve for MO effectiveness. (**C**) We standardized the subsequent screening process as much as possible. (I) For phenotype identification, we used a team-based approach that catered to the individual scientists' expertise. (II) We used specific biological assays, for example microangiography, to improve the specificity of phenotypic description. (III) We standardized written phenotypic descriptions as much as possible, using PATO-compliant terminology to enhance data communication. (IV) Finally, observed phenotypes were entered into the MODB database for organization, searchability and data sharing. (**D**) For each phenotype observed with an initial MO, validation was essential to ensure the phenotype was gene-specific and not an off-target effect. (**E** and **F**) Once a phenotype was established as gene-specific, that MO could be used to investigate new biology.

the lethal dose 50 (LD50; the dose at which ≥50% injected embryos die) for each tested MO, a useful upper limit number for subsequent testing using that particular oligonucleotide synthesis. Together, this core data set—penetrance and toxicity per dosage amount, and LD50 per MO—improves our ability to optimize phenotypic 'signal' versus off-target 'noise'.

## OFF-TARGET EFFECTS OF MOs

MO activities in zebrafish embryos include both sequence-specific RNA binding as well as effects not associated with loss of function of the targeted locus [3, 6, 17–19]. The latter 'off-target' effects can be a confounding variable when using MOs for the assignment of function to sequence in zebrafish. While the underlying mechanism of the off-target effects is unknown, one well-described and relatively consistent phenotype is p53-dependent neural toxicity [6, 17, 18] (Figure 2B, C and E, compared to Figure 2A). This effect is dose-dependent and has been estimated to be apparent in 15–20% of all MOs when injected at standard efficacy doses [3, 17]. This phenomenon was sufficiently consistent to justify systematic co-injection with a morpholino against p53 (p53 MO) to mitigate the neural toxicity (Figure 2F). One important caveat is that gene-specific, p53-dependent phenotypes will be masked using this approach [17, 19]. This effect serves as an important practical constraint on the use of MOs when studying p53-dependent processes.

A more extreme toxicity phenotype regularly noted was severe overall deformity, or what many in the zebrafish community have come to call the 'monster' phenotype (Figure 2D and E) [18, 20]. As development progresses, 'monster' embryos show one or more of the following: shortened and gnarled tails, massive body curvatures and small heads and eyes. In general, any consistently occurring phenotype observed in a screen should be carefully noted and validated: such a result likely indicates non specific toxicity of the chosen technology (see Validation section below).

## SCREENING PROCESS: PHENOTYPING ANALYSES

Morphological screening in zebrafish was pioneered through chemical mutagenesis genetic screens [8, 9]. Important lessons learned from that seminal work included the need for a process that balanced keen observation with efficient throughput effected through a team-based approach. The MO collection was initially centralized, with aliquots distributed to multiple laboratories for parallel screening work. To harness the necessary expertise within this screening approach, we organized several teams consisting of multiple laboratory members as well as internal and external collaborators, all of whom contributed a wide variety of biological interests and expertise. We distributed the work for identifying phenotypes among multiple people, decreasing the effort burden on any one person. This approach catered to the expertise in specific organ system form and function that each member of the team brought to the screen, increasing the likelihood of identifying more subtle phenotypes [Figure 1C (I)].

Gross morphological screening was based on classical forward genetic criteria [8, 9] and initially focused on morphological landmarks readily visible via a dissecting microscope. The resulting morphological screening pipeline was conducted on multiple days during the weekly screening workflow, with most screening conducted from 1 through 3 days post fertilization (dpf) based on the high efficacy of MOs during this developmental time-period [4, 6]. If a putative phenotype was noted in the first round of injections, we flagged the respective MO for re-injection confirmation, followed by validation using a second morpholino of independent sequence targeting the candidate RNA ([13]; see below).

We subsequently integrated more refined bioassays to focus screening efforts on individual organs or targeted biological pathways. For example, the Ekker and Verfaillie labs used several tools to assess vascular and blood development and function. Initially, injected embryos with largely normal gross morphology were assessed using and histological stain for blood cells on fixed embryos [21]. We later replaced this time-consuming process with functional assessment using a *fli1:GFP*; *gata1:RFP* double transgenic line which permitted simultaneous examination of vascular form and function under a fluorescent microscope [13]. This line conveniently doubled as an effective way to identify genes required for hematopoietic development [22] [Figure 1C (II)]. Parallel work in consortium laboratories was focused on identifying genes critical for other biological questions, such as lipid metabolism [13].

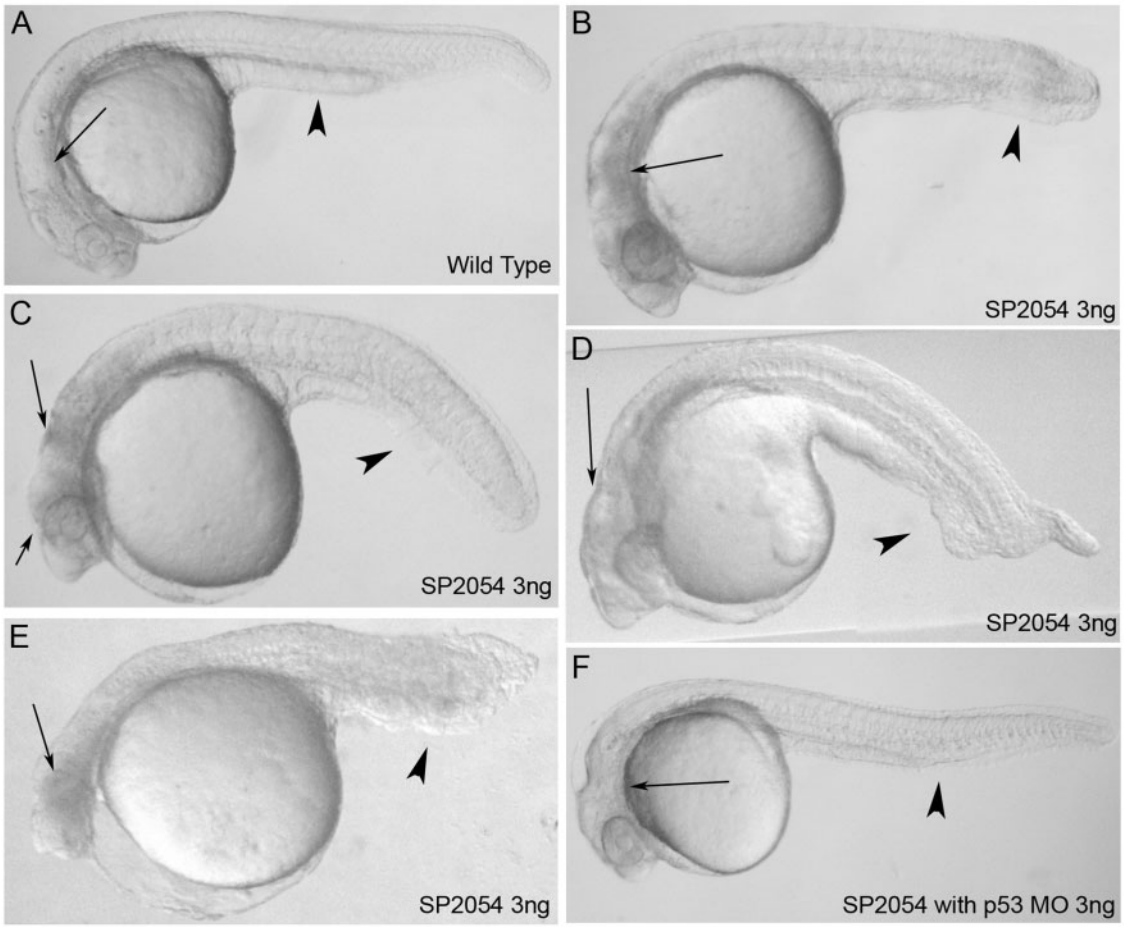


**Figure 2:** Examples of off-target effects of MOs. (**A**) A normal wild-type embryo at 24 hpf. The brain is transparent (arrow) and the tail is not bent (arrowhead). The MO used for this demonstration is targeted against SP2054 with and without p53. SP2054 MO shows a nonspecific p53 activation that does not co-localize with the endogenous expression pattern of the targeted gene [17]. (**B–E**) Examples of the standard toxicity seen in MO injected embryos that are not co-injected with the p53 MO. These four images are placed in order of increasing severity. (**B**) This embryo demonstrates some neural death, seen as a black area in the brain where it should be transparent (arrow), while the tail is somewhat bent (arrowhead). (**C**) This embryo shows more neural toxicity (arrows), and the tail is significantly bent (arrowhead). (**D**) This embryo does not show significant neural toxicity (arrow). However, gnarled tails are commonly noted due to MO toxicity (arrowhead). (**E**) This embryo shows a classic more severe, 'monster' phenotype. This embryo has p53-induced neural toxicity (arrow), but it also shows a very shortened and gnarled tail as well as small head and eyes. (**F**) Inhibition of p53 can attenuate common off-target effects. Shown is the same SP2054 MO as was used above co-injected with the p53 MO at 3 ng of each MO (therefore 6 ng of total MO). The brain is largely clear with little or no visible neural toxicity (arrow) while the tail remains fairly normal (arrowhead). The monster phenotype can be found in p53 MO co-injected embryos but usually to a lesser extent.

## SCREENING PROCESS: REPORTING THE DATA

Accurate and accessible phenotypic description was a critical component of screening success. Screening team members' instinctive response was to write detailed descriptions using personalized vocabulary based loosely on published literature and investigator experience. The major drawback to such an approach is that it facilitates neither long-term data accessibility nor comparative standards within and between screening members and screening teams. Therefore, we established a systematic approach with standardized phenotypic terminology based on Phenotype and Trait Ontology (PATO)-compliant nomenclature, an anatomically-based reference ontology [National Center for Biomedical

Ontology (NCBO) (http://www.bioontology.org/)]. We chose this method for several reasons. First, standardized terminology improved information sharing among the screening team(s) and collaborators, as well as with outside scientists, by making phenotypic descriptions readily available to those who did not examine the embryos directly. Second, having defined terminology facilitated central data collection into our Morpholino Database (MODB; [15]) and permitted machine-based assessment using both qualitative and quantitative queries. For example, team members or collaborators could search for specific phenotypes by sorting the database according to a given PATO-compliant term to find MOs with phenotypes associated with a specific organ system [Figure 1C (III)].

MODB was initially created to store and organize MOs and their corresponding observed phenotypes that were recorded after observation from records in their lab notebooks. However, we rapidly created a second-generation database to allow real-time data entry. This enabled screeners to record phenotypes at the time of observation rather than transcribing data later, reducing errors in the data transfer process and decreasing the amount of time recording phenotypes. The third-generation database made MODB [15] a web-based tool, providing for simultaneous data entry and making the results readily accessible to any scientist looking for MOs associated with a given phenotype. MODB remains online as a publicly available community tool (http://morpholinodatabase.org) [Figure 1C (IV)].

## AFTER SCREENING: PHENOTYPE VALIDATION

Any MO-induced phenotype must be validated to confirm that it is due to gene-specific effects. Several standard protocols for validating a putative morphant phenotype have been established (for detailed validation methods, see [3, 4]). First, a second MO of independent (and typically non-overlapping) sequence can be designed against the target transcript. A splice-site targeting MO [3] is now often used for this purpose because it allows the phenotype to be correlated with knockdown efficacy through quantitative measurements of altered or reduced transcript levels via quantitative real-time PCR (qRT–PCR). However, the second MO can also be a translation-blocking MO with a different sequence than the first. Regardless of the type of MO used for validation, the purpose of this experiment is to determine whether the resulting phenotypes overlap, as would be expected for independent MOs targeted to the same gene. For known genes with available antibodies, it is possible to validate efficacy with western blotting or related immunohistochemical methods to correlate reduced protein levels of the targeted gene with an observed phenotype (Figure 1D).

RNA rescue is another valuable validation method: the MO of interest and mRNA of the targeted gene (free of the MO targeting sequence) are co-injected and embryos are examined for amelioration of the gene-specific phenotype. For many genes with localized expression, MO knockdown followed by ubiquitous mRNA delivery seldom results in truly wild-type animals. Instead, the expectation is for rescue of a specific pathway or biological process within the more comprehensive *in vivo* setting.

If a mutant is available in a gene of interest, the morphant and mutant phenotypes should be compared. Validation is thus addressed where the two phenotypes agree. However, several issues may complicate a direct correlation between mutant and morphant. First, translation-blocking MOs can uncover maternally provided gene activity [6] resulting in phenotypes not noted in zygotic-only homozygous mutant animals. For some loci, generating combined maternal/zygotic mutations can circumvent this potential concern. Second, many mutations may only be hypomorphic in nature, with the resulting homozygous animals retaining some gene function. One way to address this latter concern is to conduct low-dose MO injections into heterozygous mutant embryos and compare those against injections into wild-type sibling embryos. The mutant genetic background should be permissive for the MO phenotype of interest.

We also assessed the uniqueness of the observed phenotype with respect to the overall collection. For example, an observed phenotype with a signature effect on specific aspects of biology when compared to the collected results from the hundreds of other MOs was used as corroborating evidence that the phenotype in question is likely gene-specific [13, 15]. For example, using this criterion in the secretome screen, 26/150 putative gene targets yielded likely gene-specific knockdowns [13], whereas 16 of 64 targets tested in the more focused hematopoietic screen yielded likely genes necessary for blood development [22]. In contrast, when

multiple MOs induce the same phenotype, the result is potentially an off-target effect. This correlated dataset operates as an ongoing internal control.

## USING MOS TODAY: THOUGHTS AND CAVEATS FROM OVER A DECADE OF FIELD USE

In the past decade, one noteworthy issue that sometimes affects the practical use of this reagent has been reagent storage in aqueous solution. In some cases, activity will be reduced after only a few weeks. A particularly problematic additional effect is that MO solutions can have a marked tendency to develop small precipitates that clog the needles used to deliver MOs via microinjection. Signal to noise effects can sometimes be altered in older aqueous MO aliquots. For example, increased background toxicity can be apparent with longer storage in aqueous solution. Whether these observations of an inverse relationship between MO activity and MO toxicity as storage time persists are associated with the same process is unknown. One working hypothesis is the 'increased purity' model, in which more homogeneous MO syntheses (from newer chemistries) result in a higher likelihood of insolubility issues. Indeed, the manufacturer has recommended autoclaving MOs when this phenomenon has been observed (J. Moulton, personal communication). One method we currently use for addressing the reduced shelf life has been to make separate vacuum-dried aliquots of MO and to only generate aqueous working solutions immediately before conducting microinjection experiments for phenotypic assessment. Additional efforts are ongoing in the field to address the insolubility problems.

The preferred approach in the field to distinguish specific effects from off-targeting phenotypes is now to use MOs that have available validation measurements—such as splice-altering MOs with qRT–PCR or translation-blocking MOs with antibodies. These approaches confirm the efficacy of MO targeting but do not directly address specificity. With the ever-expanding options for either identifying or generating mutants in desired genes using targeting induced local lesions in genomes (TILLING) [23–25] or custom-designed restriction endonucleases (ZFN) [26, 27], the field is more regularly turning to a reference mutant phenotype as a validation approach.

Managing off-target effects has become an increasingly important issue in dealing with MO injections: to disregard off-target issues is to potentially obscure data or to unintentionally classify unintended side-effects as genuine data. This is a particularly sensitive issue because peer reviewers from disparate fields may not have the necessary expertise to distinguish MO-induced toxicity from genuine, gene-specific MO-induced phenotypes. Therefore, we now regard co-injection with p53–MO as a crucial initial toxicity management tool [17, 19].

## MORPHOLINOS OF THE FUTURE

MOs continue to be a valuable tool for the field, and advances to this technology are still being made. For example, photocleavable caged MOs [22, 23] attach a spatiotemporal control mechanism to standard MO antisense functionality. Drawing its inspiration from the rich background of inducible gene expression technologies, this important development permits the user to confine MO activity within a region of the embryo at a chosen timepoint. Whereas first-generation MO knockdown encompasses the entire embryo, this new mechanism adds a level of control that allows for experimentation at a cellular level within an *in vivo* setting.

## CONCLUDING REMARKS

MO-based screening in zebrafish is an achievable and highly informative undertaking, provided key elements of experimental design are thoughtfully handled and tested during the prescreening phase, toxicity issues are carefully managed and responsiveness to unanticipated issues is built into the design. In our experience, harmonizing the screening process to a shared reference protocol lead to increased productivity with a reduced time burden on any particular participant, maintaining peak observational skills of the participating scientists. Furthermore, we recommend a team-based screening approach that incorporates the expertise of the scientists involved. Specifically, we standardized three critical elements of the screening process: (i) the MO dose curve to optimize phenotype recognition, (ii) the terminology used to report phenotypes to streamline data communication and (iii) the reporting format to enhance data accessibility for electronic searches using both quantitative and qualitative queries. Finally, we cannot emphasize strongly enough the necessity of careful validation of gene-specificity for each

putative morphant phenotype. We look forward to the novel biology revealed by the next generation of morpholino-based screening.

**Key Points**

- MOs can be used to functionally annotate unknown genes in zebrafish.
- Effective screening requires the use of standardized screening protocols.
- Management of off-target effects such as neural toxicity is a critical bottleneck for discerning signal from noise for phenotypic assessment.
- Validation of potential gene-specific effects is essential.

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