Guidelines for morpholino use in zebrafish: a community document

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The zebrafish (*Danio rerio*) has emerged as a powerful model to study vertebrate development and disease. Its short generation time makes it amenable to genetic manipulation and analysis, and its small size and fecundity make it especially well-suited for large-scale forward genetic and chemical screens. Fast-developing zebrafish embryos are translucent, facilitating live imaging of a variety of developmental processes in wildtype and mutant animals.

The zebrafish was originally chosen as a model with forward genetics in mind and has been used successfully in countless genetic screens. However, it quickly became a priority to be able to disrupt the function of specific genes in a targeted way. In the early 2000s, in the absence of tools for efficient targeted mutagenesis, morpholino (MO) antisense oligomers became the tool of choice for gene knock-down in a range of models including *Xenopus*, zebrafish, sea urchin and chick (Heasman et al., 2000; Nasevicius and Ekker, 2000). MOs are chemically synthesized oligomers that are typically injected into embryos at the 1-cell stage, bind complementary target mRNAs and prevent their translation or splicing. They are similar to siRNAs and shRNAs in that they interfere with gene function without disrupting genes.

Based on their ability to phenocopy known mutant phenotypes, MOs were rapidly adopted by zebrafish researchers to disrupt the function of genes that had not been uncovered by forward genetic screens. However, concerns soon arose about the off-target effects of MOs, motivating leading scientists in the zebrafish and *Xenopus* fields to publish a set of guidelines for the use of MOs including controls and rescue experiments that, if followed carefully, would help distinguish specific phenotypes from off-target effects (Eisen and Smith, 2008). Since that time, the number of zebrafish lines carrying mutations in genes of interest has grown - at first gradually, from ongoing forward genetic screens and TILLING efforts, and then exponentially with the advent of zinc finger nucleases, TALENs and CRISPRs. The ease of generating mutants now allows re-evaluation of MO-induced versus mutant phenotypes, and has led to the surprising finding that even with the careful application of the Eisen and Smith guidelines, MO-induced phenotypes can be different and often more severe than those of the corresponding mutants.

This brief document provides an updated set of guidelines regarding the use of MOs in zebrafish. We anticipate that this document will be of value for experimentalists as well as journal and grant reviewers and decision makers. These general guidelines build upon those by Eisen and Smith (2008) taking into account new developments and findings in the field, namely:

1) TALEN and CRISPR/Cas9 tools have made genome editing widely possible;
2) MO-induced phenotypes are often more severe than those of the corresponding mutants, which could be due to:

   a) phenotypic rescue of zygotic mutants by maternally provided wildtype mRNAs whose translation can be blocked by MOs,

   b) off-target effects of the MO (e.g., Kok et al., 2015),

   c) the hypomorphic nature of the mutant allele analyzed, or

d) genetic compensation in mutant but not MO-injected animals (a.k.a. morphants) (e.g., Rossi et al., 2015).

MOs are like any other knockdown reagents, and their use should thus be evaluated by journal and grant reviewers and decision makers accordingly (i.e., on a comparable footing to siRNAs, for example). However, antisense approaches are not a form of reverse genetics.

As mentioned above, the advent of TALEN and CRISPR/Cas9 tools has now made it routine to generate stable mutant lines, and hence to use reverse genetics to study gene function in zebrafish. Additionally, mutant alleles for many genes are now readily available through zebrafish community resource centers. Thus, MOs should be used alongside mutant(s) for the corresponding gene. When used in line with the following guidelines, MO-based studies can complement mutant studies, providing an additional and useful approach to our understanding of gene function.

The possibility of obtaining or generating mutants allows the morphant and mutant phenotypes to be directly compared (Fig. 1A). If they are the same, and if the MO satisfies the criteria laid out by Eisen and Smith (2008) (Fig. 1B, and see below), then the morphant can be considered an acceptable alternative to the mutant for most follow-up experiments concerning that phenotype (e.g., generating a partial loss-of-function series by using progressively lower doses of the MO, or generating a population of embryos knocked down for a specific gene product; Fig. 1C)[1].

In the case that a morphant phenotype is more severe than, or different from, a mutant phenotype, decisive experiments can be performed to distinguish between the four possible reasons listed above: i) To test the possibility that the morphant phenotype is more severe because the zygotic mutant is rescued by maternally-provided wildtype mRNAs, the morphant phenotype can be compared to the maternal-zygotic (MZ) mutant phenotype (Fig. 1D)[2]; ii) a decisive approach to determine the optimal sequence and dose of a MO that does not cause off-target effects is to inject the MO into embryos whose genome (and whose mother's genome, for maternally expressed genes) has been edited so as to eliminate the MO-binding site or to eliminate the function of the target gene (Fig. 1E). Any additional phenotype caused by the MO beyond that of the mutant is by definition an off-target effect and invalidates the use of the MO (Fig. 1F). If, however, a MO phenotype that is detected in wildtype embryos disappears or is strongly suppressed upon injection into embryos homozygous for a null[3] allele, such an observation suggests that genetic compensation may be occurring in the mutant (Fig. 1G).

In summary, a MO should be validated by comparison to, or injection into, embryos homozygous for a null allele or an allele lacking the MO-binding site. Although this latter control provides the most definitive evidence for MO specificity, we suggest the following additional guidelines for MO use[4], especially in situations in which a mutant cannot be generated[2].

A) Multiple MOs (ATG and splice blocking), or MOs and another approach (e.g., CRISPR-induced mutagenesis, or CRISPR-interference (CRISPRi)), should be used to target individual genes, and their efficiency should be assessed whenever

possible (e.g., using antibodies, RT-PCR, qPCR) to minimize the amount of MO injected.

B) The approach of validating ATG MOs by assessing their ability to suppress the expression of a co-injected target mRNA-GFP fusion is of little value, as we now know that it generally works, but it does not test the effect of the MO on the endogenous RNA. This control is no longer recommended

C) An injection control MO (standard negative control MO, 5-base mismatch MO or a suitable alternative MO) should be used to account for developmental delay. Such MOs cannot serve as controls for the specificity of the experimental MO.

D) Rescue experiments should be attempted for the approaches listed in A (e.g., by injection of mRNA or DNA lacking the MO-binding site in the case of MO studies), and if rescue is successful, control experiments should be conducted (e.g., using mutant RNA or DNA - i.e., RNA or DNA that does not encode a functional gene product).

E) Essential routine procedures include a clear dose response curve [extra caution should be exercised when one has to inject more than 5 ng of a MO to cause a phenotype (Schulte-Merker and Stainier, 2014)], the examination of statistically meaningful numbers of experimental and control animals, and extensive documentation of the penetrance and expressivity of all phenotypes.

Finally, a word of caution that previous publication of MOs is not a guarantee of their fidelity, particularly if a new phenotype is being described. These reagents should be critically evaluated to ascertain that they were properly characterized: ideally, key controls such as dose response curves and rescue experiments should be repeated when using these tools, and such data included in the manuscript.

Of course, there will be exceptions when the full set of guidelines cannot be followed and controls are not perfect; in these cases, appropriate caveats should be considered and included in the text that describes the use of the MOs, the results obtained and the conclusions drawn from them.

In conclusion, we hope that these brief, and mostly conceptual, guidelines will assist scientists working with zebrafish as well as those assessing zebrafish manuscripts and grant proposals. In addition, these guidelines may become helpful for other communities using antisense reagents to study gene function.

Footnotes

1. Caution should be exercised when conducting molecular analyses (e.g., transcriptome or proteome), as it is likely that MOs cause additional effects besides blocking the translation/splicing of target mRNAs.

2. For maternally expressed genes with essential zygotic functions, the generation of viable and fertile homozygous mutant females is not possible. In such cases, wildtype females with homozygous mutant germlines can be generated by germline replacement (Ciruna et al., 2002). If, however, the gene of interest has an essential cell-autonomous function in the development of the germline, germline replacement is not possible and thus one will not be able to directly compare the morphant and MZ mutant phenotypes; in such cases, given the current technology, gene function can only be studied using non-genetic tools (Fig. 1H).

3. Demonstrating that an allele is a null can be challenging either genetically, as very few deficiency alleles are available in zebrafish, or biochemically, as antibodies recognizing various epitopes along the protein are usually not available. Whole-gene deletions, which might also affect additional genetic elements present in introns, are more difficult to generate than small indels using current genome editing methods, and recent studies have revealed a surprising ability of the genome to recognize and circumvent putatively deleterious nonsense and frameshift mutations (Jagannathan and Bradley, 2016, Lalonde et al., 2017, Pryhozhij et al., 2017). Care should thus be taken when designing targeting tools to avoid these pitfalls.

4. Adapted from Eisen and Smith (2008), which should be consulted for a more comprehensive discussion of some of these guidelines.

References:

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., and Schier, A.F. (2002) Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc Natl Acad Sci U S A 99: 14919-14924.

Eisen, J.S., and Smith, J.C. (2008) Controlling morpholino experiments: don't stop making antisense. Development 135: 1735-1743.

Heasman J, Kofron M, Wylie C. (2000) Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. Dev Biol. 222: 124-34.

Kok, F.O., Shin, M., Ni, C., Gupta, A., Grosse, A.S., van Impel, A., Kirchmaier, B.C., Peterson-Maduro, J., Kourkoulis, G., Male, I., DeSantis, D.F., Sheppard-Tindell, S., Ebarasi, L., Betsholtz, C., Schulte-Merker, S., Wolfe, S.A., Lawson, N.D. (2015) Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. Dev Cell 32: 97-108.

Jagannathan, S., Bradley, R.K. (2016) Translational plasticity facilitates the accumulation of nonsense genetic variants in the human population. Genome research 26: 1639-1650.

Lalonde, S., Stone, O.A., Lessard, S., Lavertu, A., Desjardins, J., Beaudoin, M., Rivas, M., Stainier, D.Y.R., Lettre, G. (2017) Frameshift indels introduced by genome editing can lead to in-frame exon skipping. PloS one 12: e0178700.

Nasevicius, A. and Ekker, S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26: 216-220.

Prykhozhij, S.V., Steele, S.L., Razaghi, B., Berman, J.N. (2017). A rapid and effective method for screening, sequencing and reporter verification of engineered frameshift mutations in zebrafish. Disease models & mechanisms 10: 811-822.

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., Stainier, D.Y. (2015) Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature 524: 230-3.

Schulte-Merker, S., Stainier, D.Y. (2014) Out with the old, in with the new: reassessing morpholino knockdowns in light of genome editing technology. Development 141: 3103-4.