Response to reviewers,

We are thankful for the detailed and useful comments to our manuscript. We have carefully considered comments to improve the protocol. We believe that the quality of the manuscript benefitted from these edits, and we hope it is acceptable for publication in its current form. The more specific comments to the points raised by the editor and reviewers are listed below.

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The language of the revised manuscript was proof red before the re-submission.

1. Figure 2: Please change “sec” to “s”.

In Figure 2 “sec” was changed to “s”.

1. Please define all abbreviations before use.

All abbreviations were defined before use.

1. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Abbreviations for units were checked and changed to SI units.

1. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

The text was revised and spaces were added between numbers and their corresponding units.

1. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The ethics statement paragraph was moved before the numbered protocol steps.

1. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text was modified not to include personal pronouns.

1. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.  
   For example: Addgene# 11153, ImageJ, etc.

Commercial language was removed and products were described in the Table of Materials and Reagents.

1. 1.1 and 1.2: Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The section “1. Cloning of DNA vaccines” was rewritten with several steps (1.1. - 1.9.) and relevant references were added.

1. Lines 305-313: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Representative Results or Discussion.

All the protocol steps, including lines 305-313 (steps 8.1. – 8.4.), were modified to contain only action items. In addition, the discussion parts were moved from the protocol to the discussion section.

1. Please specify the euthanasia method.

The euthanasia method was specified in all the steps it is mentioned.

1. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing euthanasia.

The highlighted steps were changed to form a logical flow from one highlighted step to the next.

1. References: Please do not abbreviate journal titles.

References were revised according to the editor’s notes.

In addition, we added two new figures. The first one (Figure 1) represents the glass capillary needles (pulled and cut versions) and the second one is added as Supplementary Figure 1 to show the effect of the plasmid dose on the expression levels of the reporter gene. For this reason, also the numbering of the figures was changed.

**Reviewer #1:**

1. The manuscript describes the protocol for DNA vaccination and analysis in zebrafish. Unfortunately, authors have already published the abbreviated M&M as well as the results.

We were invited by JoVE to publish the described vaccination method, which was indeed based on our earlier publication (Myllymäki et al, 2017). JoVE is a method-based journal and according to our understanding, novel experimental data are not required for the publication.

1. Line 76. APC are MHC class II cells, not MCH class I.

The APC’s class was changed from I to II.

1. There is no description of the antigens cloned and how to obtain them.

The cloning protocol was described in more detail with relevant references (Steps 1.1.-1.9.).

1. Why do you use 12 µg of plasmid per fish? In salmonids it is only one. Please, give reasons.

The 12-µg dose of plasmid is based on our previous studies and on established electroporation protocols (Rao et al, 2008; Rambabu et al, 2005). We have noticed that already 0.5 µg of plasmid can be detected after electroporation with GFP ELISA, and that the expression increased as the dose increased (up to 20 µg). This data was added to the protocol as Supplementary Figure 1. For our screening purposes, high target gene expression is required to detect reporter gene expression with a fluorescence microscope and Western blot, which are necessary in confirming the correct *in vivo* translation of the target antigen. However, lower plasmid doses (0.5 - 1 µg) can be useful for other types of experimental uses. For that reason, we changed the used dose from “12µg” to “0.5-12µg” and we added the following chapter to the discussion:

“In vaccines that are used to immunize farmed fish, the plasmid dose used is typically 1µg or less 7, 33, 34. In zebrafish, reporter gene expression can also be detected at least after a 0.5 µg plasmid injection following electroporation; however, the relative target gene expression significantly increases with a higher amount of plasmid per fish (Supplementary Figure 1). In fish injected with the pCMV-EGFP reporter plasmid, injection with 5 from to up to 20 µg of plasmid resulted in 4 to 8 times higher EGFP levels in comparison to fish injected with 0.5 µg. Therefore, to ensure a high enough target gene expression, yet have injection volumes that are small enough (≤ 7 µl) to prevent excessive tissue damage or vaccine leakage, we chose to use 5 to 12 µg per fish for the preliminary screening purposes. In addition to vaccine immunogenicity, a high enough target gene expression is required to detect reporter gene expression with a fluorescence microscope and with Western blot, which are necessary for our screening purposes to confirm the correct *in vivo* translation of the target antigen. However, lower plasmid doses (0.5 - 1 µg) can be useful for other types of experimental uses. “

**Reviewer #2:**

1. The ms describes an elegant method for introduction of small volumes of vaccine containing plasmid DNA into zebrafish dorsal muscle tissue. The method is well described and tested both by antigen expression analysis (GFP) and protective potential (challenge with M.marinum).

Although this is a methods description it would be of interest to many readers what kind of antigens were used in the vaccination before challenge. Antigen 1-2-3 is not very informative.

We agree that it would be interesting for the readers to know details about the antigens. To keep the protocol as concise as possible, we decided to not include details of the antigens into the protocol. However, we added references to our latest publications, where the experimental antigens we use are explained in more detail.

**Reviewer #3:**

Major Concerns:

1. Intramuscular DNA vaccination of fish is among the most effective vaccination strategies to date. This, however, has been largely ignored by the authors in the introduction and in general in the manuscript. In generally seems that the authors are not aware of more than 20 years of research on fish DNA vaccines, nor that DNA vaccines for fish are commercially available (Apex, in Canada against IHNV virus, and more recently Clynav, Europe, against IPNV virus).

In the field of DNA vaccination, DNA vaccines targeted against fish pathogens are among the most successful ones, as indicated by commercially available fish DNA vaccines. We agree that the lessons learned from vaccination studies of aquatic cultures should be kept in mind while new approaches are developed. To emphasize this, we replaced the sentence “Currently, four DNA vaccines are licensed for veterinary practice (Tang et al, 1992; Tregoning&Kinnear, 2014; Evensen&Leong, 2013) and several are in preclinical studies.” with sentences “In mammals, a DNA vaccine against West Nile virus in horses and a therapeutic cancer vaccine for canine oral melanoma have been licensed, but are not currently in clinical use (Tregoning&Kinnear, 2014). In addition to the interest evoked in mammalian studies, DNA vaccination has turned out to be a convenient way to immunize farmed fish against viral diseases. A vaccine against fish infectious hematopoietic necrosis virus (IHNV) has been in commercial use since 2005 and a vaccine against infectious pancreatic necrosis virus (IPNV) was recently licensed (Evensen&Leong, 2013). In addition, several DNA vaccines against fish pathogens are under development.“

1. I am mentioning this because several of the comments, recommendations, and mechanisms mentioned in the introduction or in the procedure section seem to be based on mammalian background knowledge and do not necessarily apply to DNA vaccination in fish. This is also clear from the fact that an electroporation step has been included in this protocol, and this is certainly not required for any of the DNA vaccines reported to date for fish. The dose used is 12-120 times higher than the one required to vaccinate a salmon or a carp up to 10 times bigger. This is largely due to the fact that the injection procedure described is not optimal, (see comments below).

In general, I do not think this is the most optimal DNA vaccination protocol for fish, including Zebrafish. So I do not think this should be used as an example for the community to set up future DNA vaccination protocols. I am sure it works in the hands of the authors, but since JoVe aims to publish protocols that can be widely used and that are 'the best for the purpose', I am convinced that this is not the best DNA vaccination protocol. My motivations and reasoning are explained below.

The described vaccination method, including intramuscular injection and electroporation, was set up to screen the effectiveness of novel vaccine antigens before mammal studies. The aim was not provide a method to vaccinate fish in fish farms. To clarify the use of this method, in which we believe it is suitable, we modified the title: “Immunization of adult zebrafish for the preclinical screening of DNA-based vaccines”.

1. When performing DNA vaccination in any fish species, the first think to consider is to remove any air from the injection system. When we use shot-guns (for high throughput) or strippette (laboratory scale) to DNA vaccinate fish, the entire tubing or pipette tip is filled with liquid, oil or compressed air. This means that when we perform the injection, every pulse leads to a reproducible, constant and exact amount of liquid being injected. The fact that the authors use an air pump, have half of the needle filled with air, including the tubing connected to the needles, explains why they need to perform several pulses before they can inject. Having air in the system unfortunately will not allow to provide sufficient pressure to guarantee the injection of the entire dose, most of the liquid will come out of the tissue, and as soon as they withdraw the needle a few drops will come out of the needle as well. This is simple physics.

The intramuscular injection route with microinjection was selected based on our earlier experiments, where we tested the intramuscular injection with both an insulin syringe and a microinjector. Even the smallest needles used with an insulin syringe have a larger diameter than glass capillary needles, which led to larger wounds to the injection site. Rao et al. (2008) came to the same conclusion, published in Methods in Molecular Biology. In our experience, although the microinjection method is slower, the injections can be done in a more controlled manner and less leakage of the solution is observed.

The referee points out an important issue related to the use of an air pump. Several small pulses are needed to administer the vaccine dose into the muscle. However, when the fish are well anesthetized (muscle tissues are relaxed), we do not observe the leakage of the solution from the tissue. This is easily confirmed by observing, with the help of a microscope and phenol red, the administration of the vaccine. A tiny drop of the vaccine is left in the capillary to prevent the injection of air into the fish. Clearly, this is not desirably for optimizing the vaccination dose; however, the high plasmid dose enables a sufficient result for preclinical screening.

1. This also brings me to explain why they require such high doses. Generally, 1.5-2g trout, salmon or carp are fully protected by a single injection of 1 ug of plasmid DNA without the need of electroporation. 12 ug of DNA, as mentioned in this study, is far too high and perhaps the only reason why they still see antigen expression (when combined with electroporation).

See the answer above (Referee#1 Comment 4.).

1. Furthermore, some pressure is required to counteract the resistance posed by the compact muscle tissue, and contrary to what the authors suggest, pressure and a bit of tissue damage, seem to be a good ingredient to trigger a local inflammation and initiate protective responses (see references below).

Intramuscular injections cause minor tissue damage to the injection site, which most likely trigger non-specific IFN-related mechanisms, which in turn could provide a protective effect. In this protocol, fish in the control group are vaccinated with the empty pCMV-GFP plasmid, thus early vaccine responses are similar both in a control group and in the vaccine groups. In that respect, even if there was some unspecific protection, the effect is also seen in the control group, and therefore we consider this method suitable for screening purposes.

Minor Concerns:

1. Introduction: many successful DNA vaccines have been reported against fish pathogens, especially rhabdoviruses and two commercial DNA vaccines are available against fish pathogens, but none of this is mentioned, a very small selection is mentioned at the end of the comment section. Some include technical references to optimization of dose, time of challenge and immune responses triggered by DNA vaccination mostly against VHSV,

SVCV, IHNV and IPNV.

The relevant fish studies were cited in the introduction and in the discussion.

1. Furthermore, it is clear that DNA vaccines are much more effective in fish than in mammals so the mechanisms of action might be slightly different and are pathogen dependent (see references). The only route applied in fish in the i.m. route or oral route. So intradermal or subcutaneus is not an option owing to the physiology of the fish.

All experimental models for human diseases have their advantages and disadvantages. Zebrafish is in many aspects an appropriate model for many human diseases, despite of the physiological, anatomical and some immunological differences. To highlight the administration routes that are suitable for fish, we replaced the sentence in lines 64-65 with the following sentence; ” In fish studies, the most common administration routes of DNA vaccines are intraperitoneal, intramuscular and oral (Tregoning and Kinnear, 2014; Evensen and Leong, 2013; Embgrets et al, 2018), while in mammals subcutaneous and intradermal are additional options (Tregoning & Kinnear, 2014).”

1. lines: 74-79: this applies to mammals. It has been shown that the vaccine antigen-expressing cell is attacked by cells of the immune system and destroyed/eliminated. This is because it is recognized as non-self, se references number 1 and 10 below).

To specify that the described mechanisms are based on mammal studies, we modified the whole chapter:

“In mammals, DNA vaccines have been shown to stimulate different types of immune responses depending on the transfected cell types (Tregoning&Kinnear, 2014; Li&Petrovsky, 2016). Transfected myocytes secrete antigens into extracellular space or release them upon cell death, and the antigens engulfed by antigen presenting cells (APC) are subsequently presented on major histocompatibility complex II molecules2. This triggers especially CD4 and CD8 T cell responses, in addition to B cell responses (Tregoning&Kinnear, 2014; Li&Petrovsky, 2016; Cho et al, 2001). In fish, T and B lymphocytes as well as dendritic cells (DCs) have been identified, yet their division of labor in antigen presentation is less well understood (Lewis et al, 2014). Zebrafish DCs, however, have been shown to possess conserved phenotypic and functional characteristics with their mammalian counterparts (Shao et al, 2015). Furthermore, DNA vaccination has been shown to elicit similar immune responses in fish and in mammals, including T and B cell responses (Enbregets et al, 2017; Utke et al, 2008; Cuesta et al, 2010; Castro et al, 2014; Iwanami, 2014). “

In the protocol section:

1. Protocol 1.1: why should the tag be fused to the antigen? I can see that the fluorescence tag can be useful in zebrafish to track the injection site and identify antigen-expressing cells, but for this purpose (vaccination) does not have to be fused. On the contrary, it has been shown that tag fusion often hinders correct Ag conformation and thus the induction of humoral responses leading to, for example, neutralizing antibodies.

The fluorescence tag is fused to the antigen to identify the antigen expression in the injection site. It is true that the GFP tag might affect the conformation of the antigen, which may hinder humoral responses. To avoid this, we recommend confirming positive results with the same antigen without a GFP tag. For this reason, the following sentence “8.4. Confirm positive results with antigens without the GFP tag. Clone the antigens as described in steps 1 and repeat the vaccination experiment.”

1. The cloning primers besides a start codon, should also contain a Kozak sequence.

The Kozak sequence was added to the protocol step 1.4. “Include both a Kozak sequence (CCACC)27 and a start codon (ATG) in the 5’ primer. To preserve the C-terminal EGFP tag, avoid intervening stop codons (TAG, TAA, TGA) in the antigen sequence and the 3’ primer. Also ensure that the GFP tag remains in the same reading frame with the antigen of interest. “

1. Protocol 4.2: why should the vaccine mix be transferred to laboratory film and not be collected directly from a tube?

In this protocol, we load glass capillaries with long and thin loading tips. By pipetting the vaccine dose first onto the laboratory film (with a normal tip), we ensure that the whole vaccine dose is transferred to the loading tip. We have noticed that if you collect the dose directly from a tube, the total volume is lower. That is due to the surface tension in the thin capillary. If you want to transfer the whole volume from the laboratory film into the loading tip, you need to adjust the pipetting volume to approximately 1µL higher (or transfer the volume with several pipetting sets).

1. Line: 240-242: this is due to the fact that the authors are not using an air-compression-free system; air is compressible and that is why it takes a few pulses before the liquid starts to come up. Not having high pressure at the time of injection cause the liquid to accumulate at the tip of the needle, the majority of which will come out of the tissue as soon as the needle is retracted and a few drops will appear at the needle tip. This is unfortunately against the principles of dose optimization. That is perhaps why the authors need to use such high doses of the vaccine, because most of it does not enter the tissue. For DNA vaccination, an oil pump, so no air in the system, is more appropriate, the disadvantage is that current micro-oil pumps do not provide the desired high pressure for liquid release.

See comments above, major concerns 3.

1. line 246-247: actually the high pressure and the (limited) tissue damage cause by the injection are part of the factors leading to a successful DNA vaccination. First Injecting and then electroporating the fish, that is additional stress the fish do not require in case the DNA vaccination is performed properly.

We agree that minor tissue damage caused by the injection likely enhances the DNA vaccination. However, as we are working with experimental animals, it is desired to minimize the tissue damage and additional stress of the fish. It has been shown that electroporation (6 pulses, 40V/cm) is the most efficient way to transfer DNA into fish muscle (Rao et al, 2008; Rappuoli and Aderem, 2011), leading 10-fold higher expression levels compared to non-electoporated controls. The electroporation method is optimized to achieve maximal electroporation efficiency with minimal adverse effects to the fish. To explain the use of electroporation, we added the following chapter to the discussion:

“Higher antigen expression has been linked to DNA vaccine immunogenicity2. Electroporation after injection has thus been included in this protocol, as it has been shown to increase the expression of antigens or reporter genes from 4-fold to 10-fold in zebrafish32. Furthermore, electroporation as a technique causes moderate tissue injury, thus inducing local inflammation that further promotes the vaccine-induced immune responses2. On the other hand, electroporation has been generally well-tolerated. According to our experience and with our equipment, practically 100 % of adult zebrafish recover well from the 6 pulses of 40 V used in this protocol35. “

1. To reliably test vaccine efficacy at least 8 weeks is required (6 weeks is the very minimum for warm water species). 3 weeks is far too short and the authors would be looking at aspecific (type I-IFN-mediated) protection that actually confers protection against autologous and heterologous challenges. Such type I IFN is triggered by the plasmid backbone and injection (see several references below).

The misspelling “3-5 weeks” was corrected to “5 weeks” in lines 309 and 310, as it is in the Figure 5.

As the referee pointed out, it is important to know what kind of protection we are looking 5 weeks post vaccination. As shown, non-specific IFN-related mechanisms last for approximately 2-3 weeks post-vaccination (Utke et al.). Similarly, we have noticed that the expression levels of IFN type I are low 4 weeks post vaccination (Oksanen K, 2011), and therefore we assume that the detected protection 5 weeks post vaccination is not IFN-mediated. Instead, it has been shown, with a similar set up (2 doses of Ag85A-CMV vaccine and *M .marinum* infection), that antigen specific antibodies can be detected 2 weeks post vaccination and increasing concentrations of antibodies up to day 42 (Pasnik and Smith, 2005). Moreover, it has been shown in rainbow trout that transition from the innate to the adaptive responses occurs in general 3-5 weeks post DNA vaccination” (Hart et al, 2017; Lorenzen et al. 2002, 2009; McLaughan et al, 2003). For those reasons, we believe that 5 weeks is enough to test vaccine efficacy for screening purposes.

1. line 344: vary not variate

The language of the manuscript was revised.

1. Generally, 100-200 fish should be injected in no more than 1-2h and electroporation is not required. if the procedure takes so long is due to the uncertainty related to the injection and the undefined number of injections that are required before the desired amount of plasmid is injected in combination with electroporation. DNA vaccination of fish can and should go much faster. The procedure can be optimized such that the fish do not need to be under the microscope and could be kept for a very short period on the hand palm or on an agarose mole. if there is no air in the injection system and the pressure is high, one injection is more than sufficient and the fish can be placed back in the recovery tanks and the next fish can be picked up.

See answers to Major Concerns 2 and 3.

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