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TITLE:

Immunization of Adult Zebrafish for the Preclinical Screening of DNA-based Vaccines

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KEYWORDS:

DNA vaccine; vaccination; zebrafish; fluorescence imaging; pCMV-GFP plasmid; microinjector; intramuscular; pre-clinical screening; animal model; mycobacteria; antigen; immunization

SUMMARY:

Here we describe a protocol for the immunization of the adult zebrafish (*Danio rerio*) with a DNA-based vaccine and demonstrate the validation of a successful vaccination event. This method is suitable for the preclinical screening of vaccine candidates in various infection models.

ABSTRACT:

The interest in DNA-based vaccination has increased during the past two decades. DNA vaccination is based on the cloning of a sequence of a selected antigen or a combination of antigens into a plasmid, which enables a tailor-made and safe design. The administration of DNA vaccines into host cells leads to the expression of antigens that stimulate both humoral and cell-mediated immune responses. This report describes a protocol for the cloning of antigen sequences into the pCMV-EGFP plasmid, the immunization of adult zebrafish with the vaccine candidates by intramuscular microinjection, and the subsequent electroporation to improve intake. The vaccine antigens are expressed as green fluorescent protein (GFP)-fusion proteins, which allows the confirmation of the antigen expression under UV light from live fish and the quantification of expression levels of the fusion protein with ELISA, as well as their detection with

a western blot analysis. The protective effect of the vaccine candidates is tested by infecting the fish with *Mycobacterium marinum* five weeks postvaccination, followed by the quantification of the bacteria with qPCR four weeks later. Compared to mammalian preclinical screening models, this method provides a cost-effective method for the preliminary screening of novel DNA-based vaccine candidates against a mycobacterial infection. The method can be further applied to screening DNA-based vaccines against various bacterial and viral diseases.

INTRODUCTION:

The first DNA vaccine studies were performed in the 1990s[1], and since then, DNA vaccines have been tested against various infectious diseases, cancer, autoimmunity, and allergy[2]. In mammals, a DNA vaccine against West Nile virus in horses and a therapeutic cancer vaccine for canine oral melanoma have been licensed, but these are not currently in clinical use[2]. In addition to the interest evoked by 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[3]. In addition, several DNA vaccines against fish pathogens are being developed.

As traditional vaccines often contain inactivated or live attenuated pathogens, they pose a potential risk of transmitting the disease[2]. DNA vaccines, in turn, avert this risk, as they are based on the administration of plasmid encoding bacterial or viral antigens, rather than the whole pathogen itself[2,4]. DNA vaccines are produced with DNA recombination techniques, which allows the precise design of vaccine antigens and the flexible formulation of antigen combinations and adjuvants in a single vaccine construct[5]. Furthermore, the production of DNA vaccines is faster, easier and more cost-efficient than that of protein-based recombinant vaccines, which is a major advantage for vaccine candidate screening purposes, but also, for example, in the case of pandemic outbreaks[2].

In fish, the most common administration routes for DNA vaccines are intraperitoneal, intramuscular, and oral[3,6,7], while in mammals, subcutaneous and intradermal routes are additional options[2]. After an intramuscular injection, the administrated DNA plasmids enter the cells at the administration site (*e.g.*, mostly myocytes, but also resident antigen-presenting cells [APCs]). The proportion of transfected cells can be significantly increased by electroporation[2,8]. After entering the cell, some plasmid DNA enters the nucleus, where the genes encoded by the plasmid are transcribed[2]. In this protocol, we utilize the pCMV-EGFP plasmid that has a strong ubiquitous promoter optimized for eukaryotic expression[9]. In this construct, the antigens are translated as a fusion protein with a GFP. The GFP enables the confirmation of a successful vaccination and the correct antigen product by the simple visualization of antigen expression with a fluorescence microscope in live fish.

In mammals, DNA vaccines have been shown to stimulate different types of immune responses depending on the transfected cell types[2,5]. Transfected myocytes secrete antigens into the extracellular space or release them upon cell death, and the antigens engulfed by APCs are, subsequently, presented on major histocompatibility complex II molecules[2]. This triggers CD4

and CD8 T cell responses, especially, in addition to B cell responses[2,5,10]. 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[11]. Zebrafish DCs, however, have been shown to share conserved phenotypic and functional characteristics with their mammalian counterparts[12]. Furthermore, DNA vaccination has been shown to elicit similar immune responses in fish and in mammals, including T and B cell responses[6,13-16].

Both larvae and adult zebrafish are widely used to model different infectious diseases, such as the fish *M. marinum* infection model of tuberculosis used in this protocol[17-22]. In comparison with mammalian model organisms, the advantages of zebrafish include their small size, fast reproducibility, and low housing expenses[23]. These aspects make the zebrafish an ideal animal model for large-scale preclinical screening studies for novel vaccines and pharmaceutical compounds[23-25].

In this protocol, we describe how novel vaccine antigen candidates against mycobacteriosis can be evaluated by the DNA-based vaccination of adult zebrafish. First, we describe how antigens are cloned into the pCMV-EGFP expression plasmid, followed by a detailed protocol for the intramuscular injection of vaccine plasmids and the subsequent electroporation into muscle. The expression of each antigen is confirmed by fluorescence microscopy one-week postimmunization. The efficacy of the antigen candidates is then tested by experimentally infecting vaccinated fish with *M. marinum*.

PROTOCOL:

Experiments including adult zebrafish require a permission for animal experimentation for both the vaccination and the subsequent studies with the infection model. All methods and experiments described here are approved by the Animal Experiment Board of Finland (ESAVI) and the studies are carried out in accordance with EU directive 2010/63/EU.

1. Cloning of DNA Vaccine Antigens

1.1. Select an expression plasmid optimized for the eukaryotic expression of the antigen(s) of interest under a strong, constitutive promoter, such as the cytomegalovirus (CMV) immediate early promoter. To enable the *in vivo* verification of the antigen expression, select a vaccine plasmid that encodes a fluorescent tag, such as the pCMV-EGFP plasmid[9] used in this protocol.

1.2. Use appropriate web-based and bioinformatic tools to select a potentially immune-protective antigen sequence (or several sequences) of approximately 90 - 600 nt (30 - 200 aa)[26] from the gene(s)-of-interest of the pathogen that will be used in the subsequent infection model.

1.3. Use available software, or manually design primers to amplify the antigen genes from the pathogen genome and to clone the antigen sequence(s) into the multiple-cloning site of the expression plasmid. Make sure to preserve the correct reading frame when selecting the antigen.

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.

1.5. Use RNA or DNA extracted from the pathogen as a template to generate an adequate amount of PCR product with the cloning primers. Preferably, use a proofreading DNA polymerase to preserve the correct antigen sequence.

1.6. Purify the PCR product and check the correct size of the product by gel electrophoresis. Digest and ligate the PCR product with the digested vaccine plasmid.

1.7. Transform the ligation mix into competent bacterial cells according to a suitable protocol. Use an appropriate antibiotic selection for positive colonies and plasmid production in *E. coli*; the pCMV-EGFP plasmid, for example, contains an ampicillin resistance gene for this.

1.8. Use Sanger sequencing to confirm the insertion of the correct antigen sequence. The following primers can be used for sequencing antigens in the pCMV-EGFP plasmid: CMV forward 5'- CGCAAATGGGCGGTAGGCGTG-3' and EGFP-N reverse 5' CGTCGCCGTCCAGCTCGACCAG-3'.

1.9. Produce and purify a sufficient amount of the vaccine construct. Dissolve or elute the plasmid in sterile water. Make sure that the produced plasmid DNA is of high quality and the concentration is at least 0.72 µM or 2,000 ng/µL.

2. Pulling the Microinjection Needles

2.1. Prepare microinjection needles in advance. Use 10-cm aluminosilicate glass capillaries. Note that borosilicate glass capillaries are too brittle for injecting adult fish.

2.2. Pull the needles with a micropipette-needle-fabricating device.

Note: The needles should look similar to the one presented in **Figure 1**. With the device used in this protocol (**Table of Materials**), the following settings result in the desired kind of needles: heat 608, pull 250, velocity 30, time 0 (10 ms), and pressure 200.

2.2.1. Set the glass capillary in the V-groove in the puller bar and tighten the clamping knob lightly.

2.2.2. Move the holder next to the filament and gently push the capillary through the filament into the puller bar on the other side of the filament. Avoid touching the filament with the capillary.

2.2.3. Tighten the clamping knobs, set down the safety glass, and press the pull button.

CAUTION: The filament is hot.

2.3. Place the needles on a piece of reusable adhesive inside a 15-cm Petri dish plate to protect the needle tips. Keep the dish covered to keep the needles clean.

3. Filling the Micropipette Needles

3.1. Prepare the vaccine mix. Use 0.5 - 12 µg of plasmid per dose. If combining several different plasmids in one vaccination mix, use a maximum total DNA concentration of 12 µg per fish.

3.2. Calculate the volume of the vaccine "master mix" according to the number of fish in each group (see below). Add 1 µL of sterile-filtered phenol red to ease both the filling of the capillary needles and the observation of the injection. Fill the needle with sterile 1x PBS up to a maximum total volume of 5 - 7 µL[8].

Note: Injection volumes higher than 7 µL can result in the occasional leakage of the injection solution and should, therefore, be avoided.

3.3. Place a piece of tape, glue side up, on an appropriate holder, for example, the side of an empty tip box. Gently attach the capillary needles to the tape.

3.4. Pipet a maximum of 7 µL of the vaccine mix onto a piece of laboratory film. Using a loading tip, transfer the vaccine from the film into the needle. Pipet slowly and carefully, avoid pipetting air bubbles into the needle.

3.5. Let the needles settle for 15 - 30 min to remove possible remaining small air bubbles.

4. Setting the Microinjector and Electroporator for Immunization

4.1. Set the micromanipulator and a light source into the right position. Switch the air pressure tap to the open position.

4.2. Adjust the parameters for the pneumatic pump (see also **Figure 2**) as follows.

4.2.1. Set the **vent** knob on the **Eject port** to **hold** to prevent backfilling of the pipette by capillary action. Set the **tubing** from the eject port to the micropipette. Do not use the Vacuum port in this protocol.

4.2.2. Adjust the **pulse length**: use the **timed mode**, where an electronic timer controls the duration of the time the pressure solenoid stays open. Check that the green lamp next to the Eject pressure gauge illuminates when the pressure solenoid is open (energized).

4.2.3. Set the pulse **range** to 10 s; with this setting, the pulses may be further set from 100 ms to 10.1 s. Use the **10-turn period dial** for fine-tuning the pulse length—every turn of the dial is 1.0 s. If needed, this can also be adjusted during an injection.

4.2.4. Use the pulse initiator ("start button") on the front panel of the pneumatic pump, or a remote **foot switch** (recommended). This is connected to the front panel of the pneumatic pump.

4.3. Set the needle onto the micropipette holder of the micromanipulator. Cut the tip of the needle with tweezers so that liquid can be pushed out, and use the microscope to view the correct position. Press the foot switch once to see that a 1-s pulse pushes a small droplet out of the needle.

4.4. Use the following settings for the **electroporator**: voltage = 40 V; pulse length = 50 ms, number of pulses = 6. Connect the tweezers to the electroporator. See that the actual voltage and pulse length shown on the monitor do not differ significantly from the settings.

5. Injection of the DNA Vaccine and Electroporation

5.1. For immunizations according to this protocol, use healthy, 6- to 12-month-old adult zebrafish. Keep the fish in a flow-through system with a 14/10-h light/dark cycle, with a maximum of seven fish per 1 L of water and feed the fish normally.

5.2. Prepare a 0.02% 3-aminobenzoic acid ethyl ester (tricaine) solution (pH 7) in tank water for anesthetizing the fish[28]. Use a 10-cm Petri dish or something similar.

5.3. Prepare a recovery tank by filling a 5-L beaker with 3 L of clean system water.

5.4. Prepare a vaccination padding to keep the fish in a fixed position during the vaccination. Take a 5 x 7 cm piece of a 2- to 3-cm-thick sponge. Cut a groove into the sponge with a scalpel blade or sharp scissors.

Note: The same vaccination padding can be used in multiple experiments. Disinfect the sponge between the experiments by soaking it in 70% ethyl alcohol and allow to dry.

5.5. Thoroughly soak the sponge in the system water and set the sponge on a Petri dish.

5.6. Fast the fish 24 h before the vaccinations.

5.7. Anesthetize one zebrafish by placing it on a Petri dish containing 0.02% tricaine. Wait until the fish does not respond to touch stimulation and until there is no movement of the gills. Anesthetize a single fish at a time.

5.8. Using a plastic spoon, transfer the anesthetized zebrafish onto the wet sponge and set the fish's ventral side down into the groove. In the correct position, ensure that the head and most of the body of the fish are inside the groove and the dorsal fin and the tail are protruding out from the groove.

5.9. Under the microscope, carefully place the needle in an approximately 45° angle close to the zebrafish's dorsal muscle, using the x- and y-axis fine-tuning wheels on the micromanipulator.

5.10. Find the small spot without scales in front of the dorsal fin, where pushing the needle does not demand force. If resistance is felt, try an adjacent spot. Avoid injuring the spine, the dorsal fin, or the scales.

Note: If the needle bends while pushing, shorten the needle slightly by cutting it.

5.11. Use the foot switch to gradually inject the vaccine solution into the muscle in intervals of a few seconds. Observe the injection through the microscope: phenol red is visible as it enters the muscle tissue. Adjust the duration of the pulse if needed.

Note: Alternatively, use the pulse initiator button on the front panel of the pneumatic pump for the injection. However, the use of a remote foot switch allows using the other hand for adjusting the pulse length by the 10-turn dial wheel. Avoid injecting the solution too fast, since this may cause excessive tissue damage. Make sure not to inject any air.

5.12. Electroporate the fish immediately after the injection. Make sure that the fish is still under anesthesia. Keep the fish on the sponge and set the fish between tweezer-type electrodes, so that the electrodes are located on each side of the injection site. Do not press the electrode tweezers too tight but keep both electrodes in contact with the fish.

5.12.1. Press the start button on the electroporator to give six 40-V, 50-ms pulses.

5.12.2. Gently transfer the fish to the recovery tank.

5.12.3. Clean the electrodes after each electroporation by swiping them with 70% ethanol.

Note: Carefully monitor the well-being of the fish after the vaccination. Euthanize any fish showing signs of discomfort (a slow recovery from anesthesia, aberrant swimming, gasping) in 0.04% tricaine. After recovery, transfer the fish to the flow-through unit and feed it normally.

6. Visualization and Imaging of Antigen Expression

6.1. Anesthetize the fish in 0.02 % tricaine 2 - 7 d after immunization, and use a UV-light to see EGFP expression near the injection site.

6.2. To capture images, use a fluorescence microscope to visualize EGFP expression at the injection site[8]. Use a 2X objective lens and select the correct filter to visualize fluorescence or visible light views.

6.3. Keep the anesthetized fish still by pressing the ventral fin gently with tweezers toward a Petri dish bottom. Take both light-microscope and fluorescence images of the same area.

6.4. Merge the light-microscope and fluorescence images using the appropriate software[29]. Add a scale bar.

Note: Visual inspection under a UV light is an easy and non-invasive operation that is suitable for the routine verification of successful vaccinations, also in large-scale experiments. If no images of the fish are required, this step can also be performed in regular fish tanks without the need to anesthetize or move the fish.

7. Quantification of the Expression Level and Size of the Antigens

7.1. Euthanize the fish in 0.04% tricaine. Dissect the fluorescent part of the dorsal muscle with a scalpel and tweezers under UV-light. Extract proteins from the samples[8].

7.2. Verify the correct size of *in vivo*-produced proteins with a western blot analysis, using a horseradish peroxidase (HRP)-conjugated GFP antibody (or similar)[8]. Include a negative control (unimmunized fish) to exclude any background signals and unspecific binding, together with a control expressing EGFP without a fused antigen.

7.3. Quantify the expression of each antigen using a GFP-ELISA[8] (optional).

Note: For the western blot analysis, the expected size (in kDa) of the antigen-fusion proteins can be calculated, for example, by the equation:

Molecular Weight (MW) of dsDNA = (number of nucleotides x 607.4) + 157.9; or by using web-based tools.

8. Combining the Vaccination Protocol with an *M. marinum* Infection Model

8.1. Determine the group size required for the reliable determination of the effectiveness of a novel vaccine candidate in the infection model and the assay used (see, for example, Myllymaki *et al*.[24] and Charan and Kantharia[30]). Carry out the appropriate power calculations while planning the experiments.

8.2. To evaluate the effectiveness of vaccine candidates, infect the fish 5 weeks postvaccination. Use an intraperitoneal infection with approximately 30 colony-forming units (cfu) of *M. marinum*, which leads to a latent infection in most fish[8,20,31].

Note: When using *M. marinum*, follow a BSL2 safety protocol. The preparation of the bacterium or virus depends on the pathogen.

8.3. Quantify the number of pathogens in each fish. Euthanize the fish 4 weeks postinfection and determine the bacterial burden in each fish from the extracted DNA with qPCR using primers specific for *M. marinum*[20,24].

Note: Be sure to include an appropriate control group and use the correct statistical methods for analyzing the results. Generally, a group of fish immunized with the empty pCMV-EGFP plasmid is a suitable negative control.

8.4. Confirm positive results with antigens without the GFP tag. Clone the antigens as described in step 1 and repeat the vaccination experiment.

REPRESENTATIVE RESULTS:

The steps involved in the DNA vaccination protocol of adult zebrafish are illustrated in **Figure 3**. At first, the selected antigen sequences are cloned into a pCMV-EGFP plasmid and plasmid DNA is produced and purified[24] (**Figure 3**). Vaccine candidates are then injected intramuscularly with a microinjector and the injection site is electroporated to improve the intake of the plasmid into cells (**Figure 3**). The used vaccination dose was optimized by injecting different amounts of the pCMV-EGFP plasmid and measuring the GFP expression with ELISA (**Supplementary Figure 1**). Two to seven days postvaccination, the expression of the fusion protein is detected under UV light and visualized with fluorescence microscopy (**Figures 3** and **4**). The expression levels of different antigens may vary from very intensive (antigen 1) to a faint expression (**Figure 4**). In addition, GFP expression can be observed across the dorsal muscle (antigen 1), or in a more limited area (antigen 2) (**Figure 4**). However, if no fluorescence is detected within 10 days, it is recommended to make sure that there are no mistakes in antigen cloning or primer design. To confirm that the expressed fusion protein is of the correct size, proteins can be extracted from the muscle tissue around the injection site and used for a western blot analysis.

The effect of the vaccine candidates is evaluated by challenging the fish with a low dose of *M. marinum* by an intraperitoneal injection (**Figure 5**). Four to five weeks postinfection, the bacterial counts are determined with qPCR and compared to bacterial loads in the control group (**Figure 5**). Furthermore, the effectiveness of the most promising vaccine candidates can be tested by monitoring the survival after a high dose *M. marinum* infection (**Figure 5**). However, in addition to giving a quantitative result on the progression of the infection, instead of merely a status of alive or dead, the qPCR-based cfu quantification requires less time and smaller group sizes and is, therefore, a more ethical approach for a primary screen. Overall, this protocol facilitates the screening of the effectiveness of novel vaccine antigens within 12 weeks (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Close-up (12x) of aluminosilicate needles used in the adult zebrafish *intra muscular* injections. The tip below has been cut with tweezers and is ready to be used for microinjections.

This figure has been adapted from Oksanen[35].

Figure 2: Microinjection equipment and set-up. The main components of the equipment needed for the DNA vaccination of adult zebrafish are highlighted in bold. The critical adjustments are indicated.

Figure 3: Preparing the DNA vaccine plasmids and the immunization procedure. (**1**) Selected antigens are cloned adjacent to the GFP tag in the pCMV-EGFP plasmid. (**2**) The vaccine construct is produced microbiologically, concentrated, and purified. (**3**) 12 µg of plasmid is injected into the dorsal muscle of an anesthetized adult zebrafish with a microinjector, and the injection site is subsequently electroporated with six 40-V, 50-ms pulses. (**4**) Two to seven days postvaccination, the GFP expression of the antigen-GFP fusion protein is visualized with a fluorescence microscope. (**5**) The fluorescent part of the dorsal muscle can be dissected and used for protein extraction. The size of the fusion protein is confirmed with a western blot analysis and the expression level with GFP-ELISA.

Figure 4: Visualizing the expression of the antigen-EGFP fusion protein. Anesthetized adult zebrafish are vaccinated with 12 µg of experimental vaccine antigens (antigen 1 - 3) and the injection site is electroporated, subsequently, with six 40-V, 50-ms pulses. Two to seven days postvaccination, the injection site is imaged with a microscope. First, the expression of GFP is detected under a fluorescence microscope. The area is inspected using a 2X magnitude objective and imaged and saved in .tiff form. The light microscope image of the same area is merged with the fluorescence image using the ImageJ software. The quantity and position of the antigen expression may vary between antigens and individual fish. For example, the expression of antigen 1 is observed across the dorsal muscle and the expression of antigen 2 is seen as small spots, whereas antigen 3 is strongly expressed in a more limited area.

Figure 5: Testing the effectiveness of vaccine candidates against a mycobacterial infection. (**1**) Adult zebrafish are vaccinated with experimental DNA vaccines against mycobacteriosis. (**2**) Five weeks postvaccination, the fish are infected with a low dose of *Mycobacterium marinum* (~30 cfu). (**3**) Four weeks later, the internal organs are dissected and used for DNA extraction. (**4**) The bacterial count in each fish is quantified with qPCR using *M. marinum*-specific primers. Immunization with antigen 1 led to a significant decrease in the bacterial counts ($p < 0.01$, two-way ANOVA), while antigens 2 and 3 had no effect. (**5**) The protective effect of the most promising vaccine candidate (antigen 1) is further evaluated in a survival experiment, where fish are infected with a high dose (~10,000 cfu) of bacteria and their survival is monitored for 12 weeks. Consistent with the decrease in the bacterial burden observed in panel **4**, vaccination with antigen 1 also improved the survival of the fish upon an *M. marinum* infection ($p < 0.01$), suggesting this antigen could be a promising candidate for a novel vaccine against tuberculosis.

Supplementary Figure 1: Amount of plasmid DNA affects plasmid-derived EGFP expression in adult zebrafish. Groups of fish ($n = 5$ in each group) were injected with 0.5 - 20 µg of pCMV-EGFP, and electroporation (six pulses of 50 V) was used to enhance the transfection. Control fish (CTRL) were injected with 2 µg of the empty pCMV plasmid not containing the EGFP gene. GFP-ELISA

was performed 3 days postinjection to define the relative EGFP expression in fish homogenates. P-values: *$p < 0.03$, **$p < 0.004$. The error-bars represent standard deviations. NS = not significant. This figure has been adapted from Oksanen[35].

DISCUSSION:

The procedure of immunizing adult zebrafish with DNA-based vaccines requires some technical expertise. Even for an experienced researcher, vaccinating a single fish takes approximately 3 minutes, excluding preparations. Thus, a maximum of roughly 100 fish can be immunized within a day. If more than 100 fish are required for the experiment, the immunizations can be divided between up to 3 days. In addition to the quality of the experiment, sufficient training of the researcher(s) for handling the fish and performing the immunization is essential for the well-being of the fish. Make sure to follow local legal and animal welfare rules and guidelines when it comes to housing the fish, planning the experiments, and the qualifications required for the personnel carrying out the experiments.

In summary, there are several critical steps to avoid complications in the immunization protocol. For the successful immunization, ensure that **1)** the fish to be immunized are healthy and sufficient in age and size (the immunization of more juvenile fish can require down-scaling the vaccine volume and the electroporation settings); **2)** the fish are properly anesthetized with tricaine no stronger than 0.02% 3-aminobenzoic acid ethyl ester, and they remain anesthetized throughout the entire procedure (anesthesia should be kept as short as possible to ensure the recovery of the fish); **3)** the sponge paddle is properly soaked; **4)** liquid is injected in each pulse from the pneumatic pump and, if not, the pulse length is adjusted (pulling the needle slightly backwards along the y-axis can help); **5)** there are no air bubbles with the vaccine solution; **6)** the electroporation settings and the actual pulse voltage and length are correct; **7)** the electrodes do not cause skin damage on the electroporation site (during the electroporation, keep the electrodes in gentle contact with the fish, and release the fish immediately into the recovery tank after electroporation).

It is important to monitor the fish after the electroporation in the recovery tank and to euthanize any fish showing signs of discomfort. Furthermore, it is necessary to practice the procedure before starting a large-scale experiment, to ensure a fluent workflow. If possible, ask a sufficiently trained colleague for assistance with filling the needles and the electroporation.

The DNA vaccination method enables the tailor-made design of vaccine antigens. It is possible to clone the whole antigen or, preferably, select parts of the antigen based on cellular localization and immunogenicity[24]. In addition, the method enables combining several antigens or adjuvants into one vaccine construct or injecting several separate plasmids at the same time[2]. By including a stop codon after the antigen sequence or by excising the EGFP gene from the plasmid, it is possible to utilize the same plasmid vector also to express the antigen without the subsequent N-terminal GFP tag. This may be reasonable in confirming the positive screening results, as the relatively large size of GFP can affect the folding of the antigen and, thus, restrict humoral responses potentially evoked by the vaccination.

A higher antigen expression has been linked to DNA vaccine immunogenicity[2]. 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 fourfold to tenfold in zebrafish[32]. Furthermore, electroporation as a technique causes moderate tissue injury, thus inducing local inflammation that further promotes the vaccine-induced immune responses[2]. On the other hand, electroporation is generally well-tolerated. With the equipment used here, practically 100% of adult zebrafish will recover well from the six pulses of 40 V used in this protocol[35].

In addition to using electroporation to enhance the entry of the vaccine plasmid into the cells, we use a strong ubiquitous promoter in the vaccine plasmid and a polyA tail at the 3' end of the antigen to improve antigen expression in the transfected fish cells. In some cases, if the codon usage of the target pathogen significantly differs from the vaccinated species, codon optimization has been found useful in further increasing target gene expression[2]. In this zebrafish–*M. marinum* model, however, codon optimization had no significant effect on the expression levels of two mycobacterial model genes, *ESAT-6* and *CFP-10,* and has, thus, been deemed unnecessary in this model[35].

Target gene expression profiles have some temporal variation between the antigens, depending, for instance, on the size and the structure of the antigens in question. However, antigen expression is usually similar within a group of fish immunized with the same vaccine. Typically, the brightest EGFP expression is observed four days to one-week postvaccination, but a scale of 2 - 10 days is possible. It is recommended to validate the expression of each antigen-EGFP fusion protein in a small group of fish (2 - 3) before including the antigen in a large-scale experiment. If no GFP expression is observed at any point 2 - 10 days after immunization, make sure that **1)** the immunization protocol was carefully followed. Always have a group of fish immunized with the empty pCMV-EGFP plasmid as a positive control and make sure that **2)** the antigen design and molecular cloning was carried out correctly (adequate primer design; the antigen and the EGFP tag are both in the same reading frame and no intervening stop codons are included). In some cases, despite the correct antigen design, GFP cannot be detected. This may be due to the incorrect folding or rapid breakdown of the fusion protein. In these circumstances, it may be necessary to redesign the antigen.

In vaccines that are used to immunize farmed fish, the plasmid dose used is typically 1 µg or less[7,33,34]. In zebrafish, reporter gene expression can also be detected after at least 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, an injection with 5 - 20 µg of plasmid resulted in four to eight times higher EGFP levels in comparison with 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 any excess 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 is necessary for 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.

In conclusion, this protocol for the immunization of adult zebrafish with a DNA plasmid can be used in the preclinical testing of novel vaccine candidates against various bacterial or viral infections. The expression of the vaccine antigen as a GFP-fusion protein allows the visualization of a successful immunization event and antigen expression. We apply this method for the preclinical screening of novel vaccine antigen candidates against tuberculosis. For this, we infect the zebrafish five weeks postvaccination and determine the bacterial counts in each fish with qPCR[20,24].

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DISCLOSURES:

The authors have nothing to disclose.

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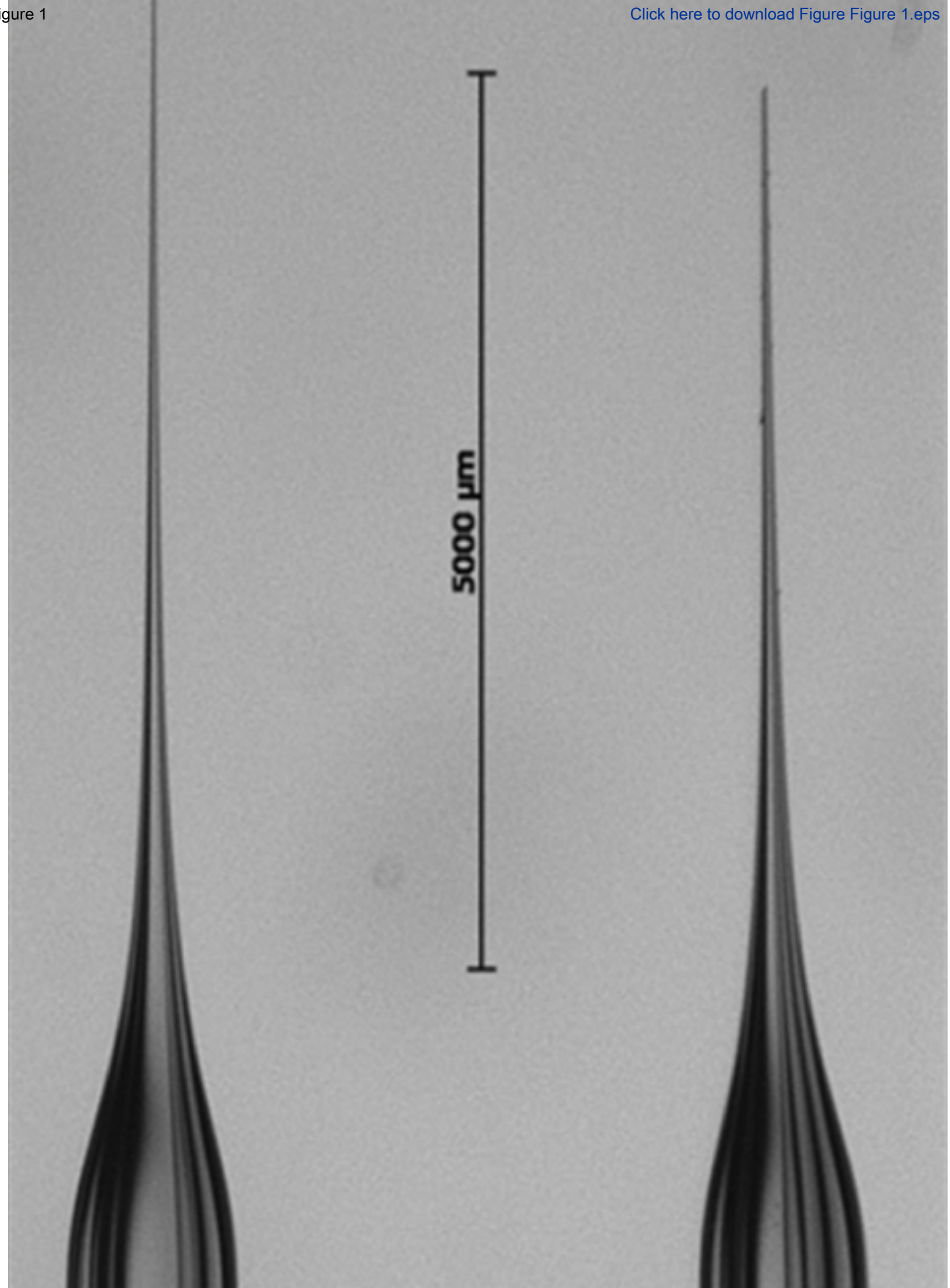

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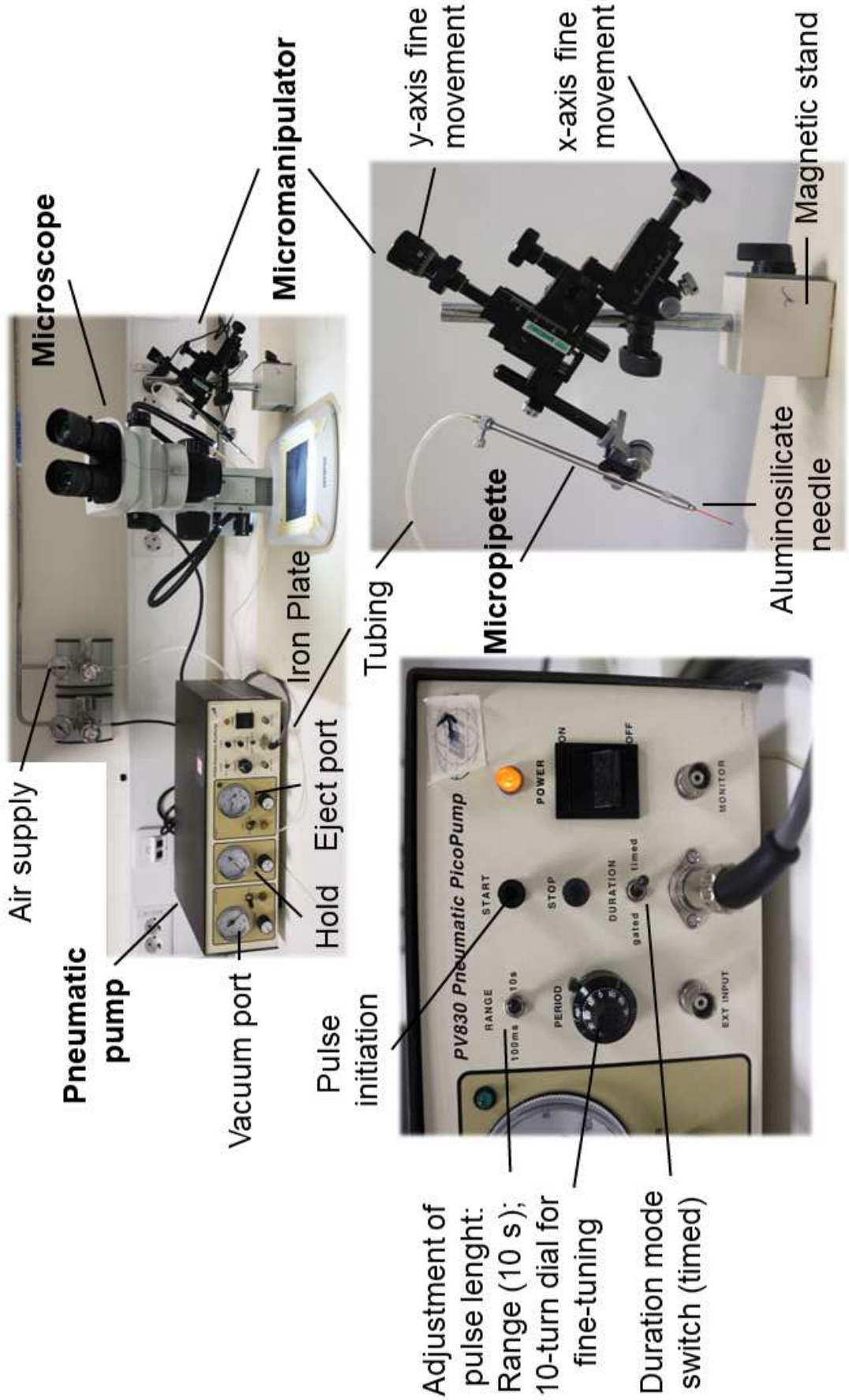
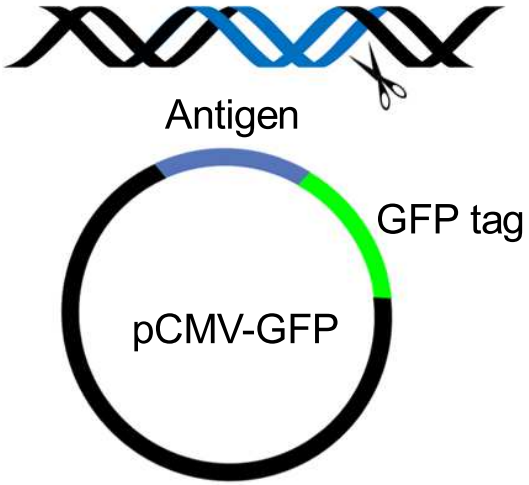
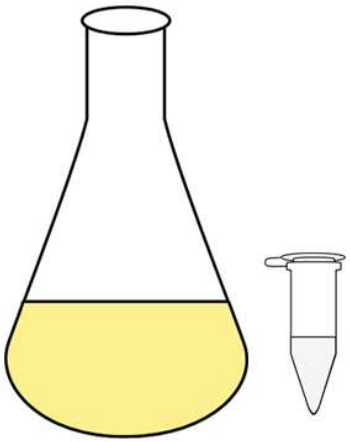


Figure 3

1. Preparing the vaccine construct by cloning the antigen into the plasmid

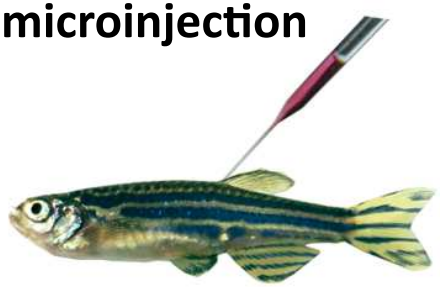


2. Production and purification of the vaccination plasmid



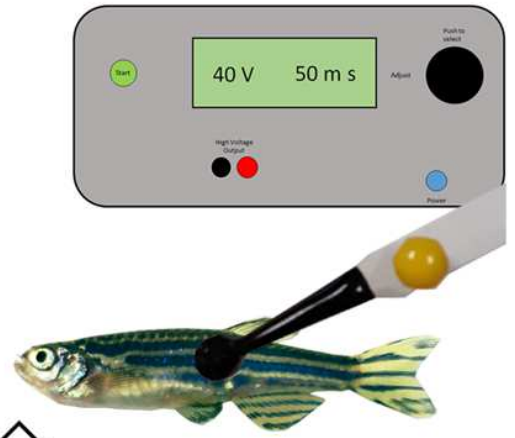
3. Vaccination

Intramuscular microinjection



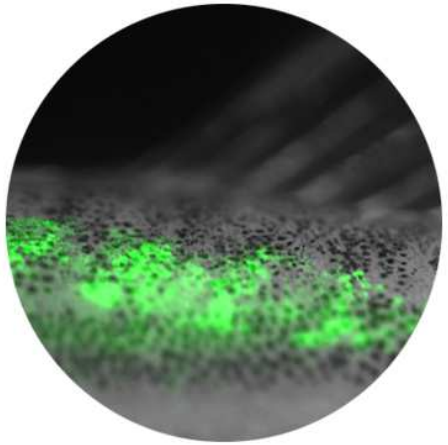
+

Electroporation



5-7 days

4. Visualizing the expression of the antigen-GFP fusion protein



5. Protein extraction and Western blot analysis / ELISA

Figure 4

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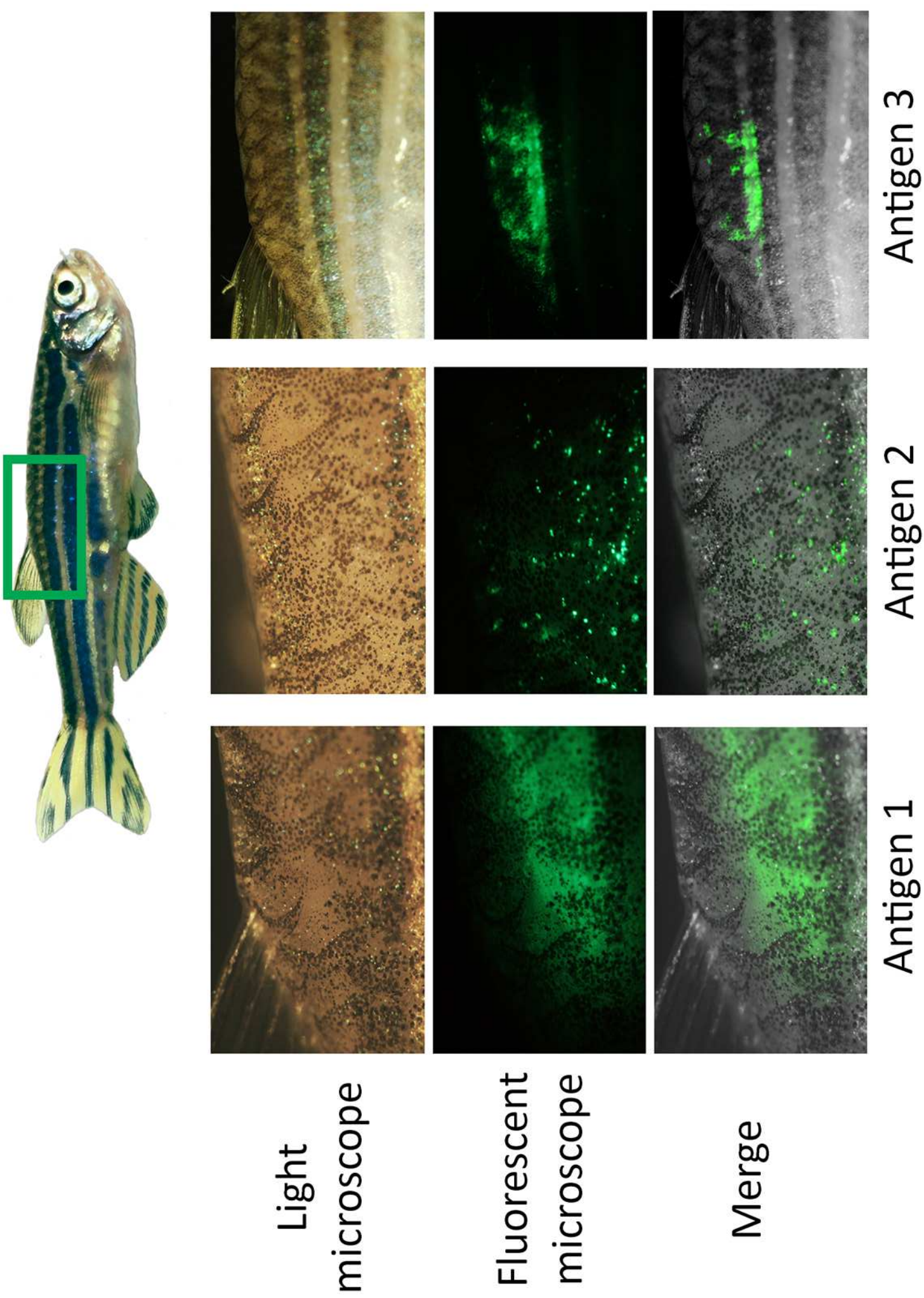
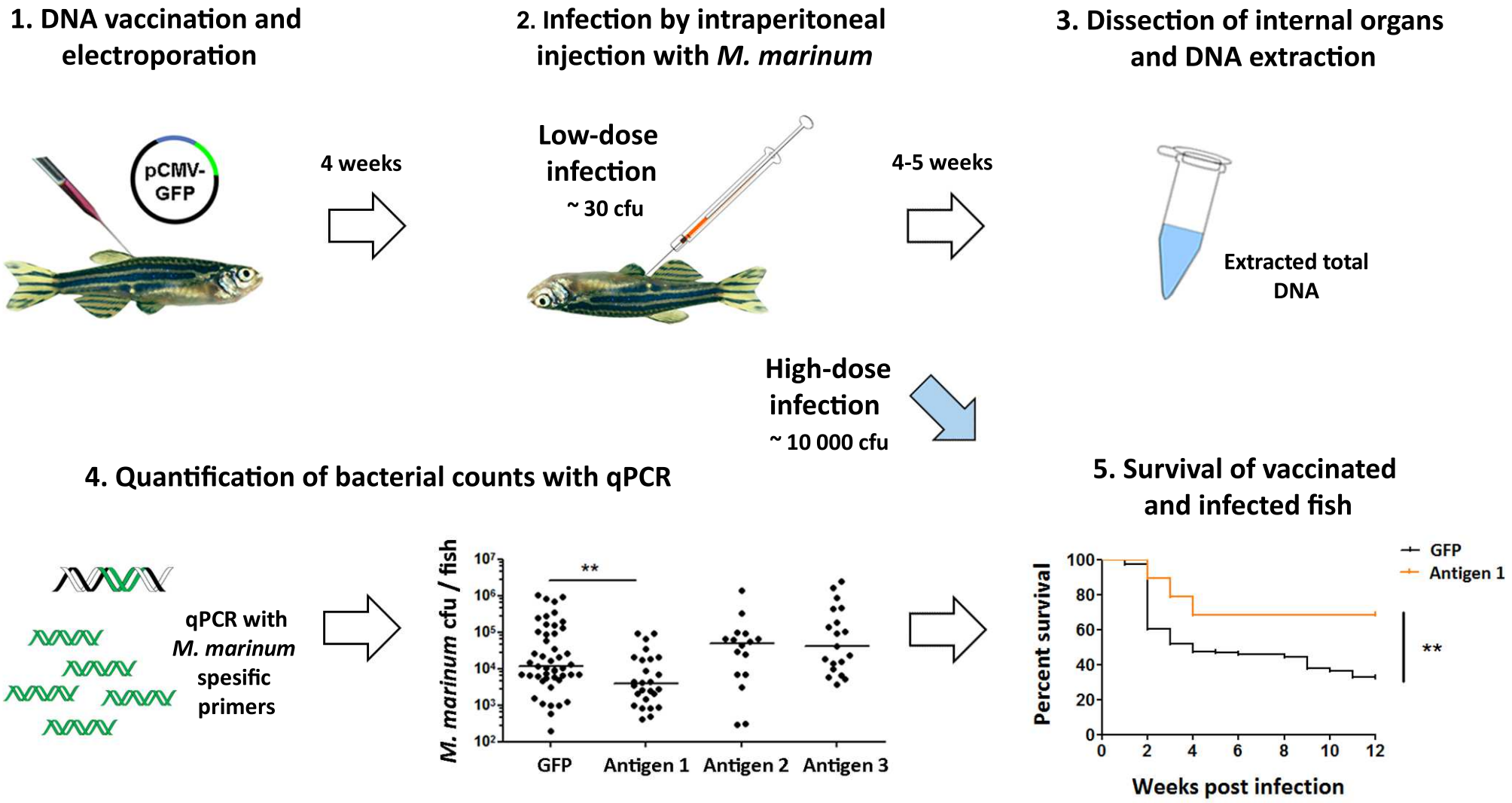


Figure 5



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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
pCMV-GFP plasmid	Addgene	#11153	
2-propanol	Sigma-Aldrich	278475-2L	DNA extraction
Ampicillin sodium salt	Sigma-Aldrich	A0166-5G	
Chloroform	Merck	1.02445.2500	DNA extraction
ECM Electro Square Porator	BTX Harvard apparatus	BTX ECM 830	
FastPrep-24 5G	MP Biomedicals	116005500	homogenizer
Flaming/brown micropipette puller	Sutter Instrument Co.	P-97	Pulling of needles
GeneJet PCR Purification kit	ThermoFischer Scientific	K0701	
GFP ELISA kit	Cell Biolabs, Inc.	AKR-121	
Guanidine thiocyanate (FW 118.2)	Sigma-Aldrich	G9277-500G	DNA extraction
ImageJ2	imagej.net/Downloads		freely available software
LB Agar	Sigma	L2897-1kg	
LB Broth (Miller)	Sigma	L3522-1kg	
Micromanipulator	Narishige	MA-153	
Microscope	Nikon	AZ100	fluorescense microscope
Microscope	Olympus	ZS61	
Nightsea Full adapter system w/Royal Blue Color light head	Electron Microscopy Sciences	SFA-RB	
PBS tablets	VWR Chemicals	E404-200TABL.	
Phenol red sodium salt	Sigma-Aldrich	114537-5G	
PV830 Phneumatic Pico Pump	WPI	SYS-PV830	
QIAGEN Plasmid Maxi kit	Qiagen	ID:12163	plasmid extraction
Sodium citrate (FW294.1)	VWR Chemicals	27833.294	DNA extraction
Tri Reagent	Molecular Research Center, Inc.	TR 118	DNA extraction
Tricaine (ethyl 3-aminobenzoate methanesulfonate salt)	Sigma	A5040-100g	anestesia and euthanasia sc
Tris (free base) (FW121.14)	VWR Life Science	0497-500G	DNA extraction
Tweezertrodes Electrodes (7mm) Kits	BTX Harvard apparatus	BTX 450165	tweezer type electrodes
2.8 mmCeramic beads	Omni International	19-646-3	DNA extraction
2ml Tough tubes with caps	Omni International	19-649	DNA extraction
Aluminosilicate capillaries	Harvard apparatus	30-0108	
Microloader 20 µl	eppendorf	5242956.003	loading tips
Petri dishes, 16 mm	Sarsted	82.1473	

Scalpels	Swan Morton	0501	
Parafilm	Bemis		laboratory film
Pins			
Plastic spoon			
Spatula			
Sponge			
Styrofoam workbench			
Tweezers			

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8. 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.

9. 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.

10. 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.

11. Please specify the euthanasia method.

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

12. 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.

13. 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.

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

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

3. 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.).

4. 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."

2. 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”.

3. 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.

4. 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.).

5. 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.

2. 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).”

3. 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 molecules[2]. 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:

4. 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."

5. 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. "

6. 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).

7. 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.

8. 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 immunogenicity[2]. 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 zebrafish[32]. Furthermore, electroporation as a technique causes moderate tissue injury, thus inducing local inflammation that further promotes the vaccine-induced immune responses[2]. 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 protocol[35]. "

9. 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.

10. line 344: vary not variate

The language of the manuscript was revised.

11. 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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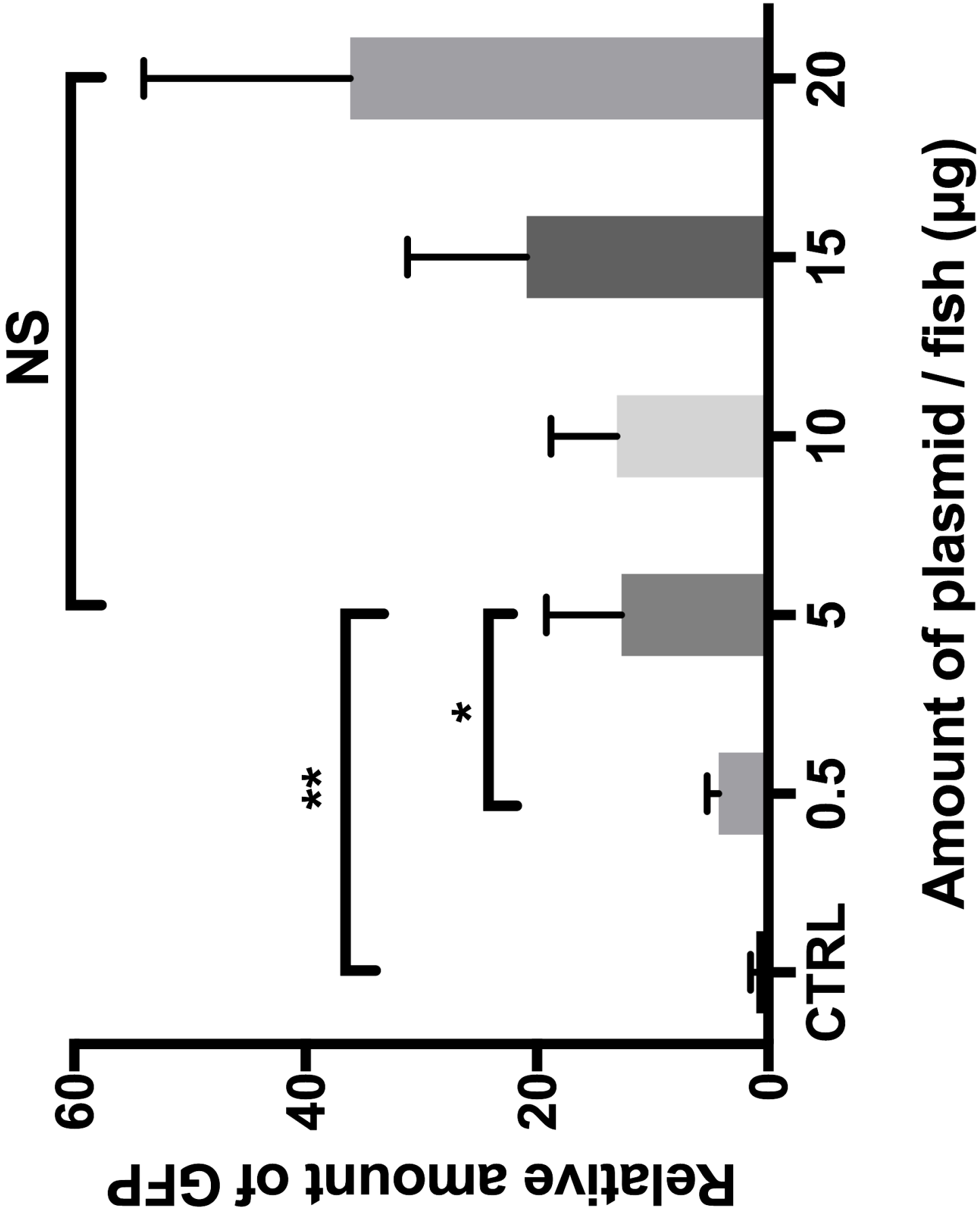

Vineeta Bajaj <vineeta.bajaj@jove.com>

Re: Response Requested: JoVE Submission JoVE58453R2 - [EMID:e81f3bbb1c99164b]

Mika Rämet <mika.ramet@uta.fi> Wed, Aug 1, 2018 at 4:17 AM
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