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Developing systems for high-throughput screening of infectious diseases using zebrafish

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Chapter 3

3

Establishment and optimization of a high-throughput setup to study *Staphylococcus epidermidis* and *Mycobacterium marinum* infection as a model for drug discovery

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Abstract

Zebrafish are becoming a valuable tool in the preclinical phase of drug discovery screenings as a whole animal model with high-throughput screening possibilities. They can be used to bridge the gap between cell based assays at earlier stages and *in vivo* validation in mammalian models, reducing, in this way, the number of compounds passing through to testing on the much more expensive rodent models. In this light, in the present manuscript is described a new high-throughput pipeline using zebrafish as *in vivo* model system for the study of *Staphylococcus epidermidis* and *Mycobacterium marinum* infection. This setup allows the generation and analysis of large number of synchronous embryos homogenously infected. Moreover the flexibility of the pipeline allows the user to easily implement other platforms to improve the resolution of the analysis when needed. The combination of the zebrafish together with innovative high-throughput technologies opens the field of drug testing and discovery to new possibilities not only because of the strength of using a whole animal model but also because of the large number of transgenic lines available that can be used to decipher the mode of action of new compounds.

Introduction

To date the zebrafish (*Danio rerio*) has been successfully established as an efficient model to study a variety of infectious diseases [1]. The zebrafish embryo offers unique *in vivo* imaging possibilities due to their transparency and the large number of existing transgenic reporter lines expressing fluorescent proteins. This powerful combination makes it possible to track different immune cell types in time while interacting with pathogens such as *Mycobacterium marinum*, the closest relative of *M. tuberculosis* [2], or *Staphylococcus epidermidis*, the main causative of biomaterial-associated infection [3-5]. Different routes of infection can be used in zebrafish embryos depending on the purposes of the study [6].

One of these infection routes is yolk injection of the bacteria. The main advantage of this method compared to the others is that yolk infection can be performed automatically via robotic injection, significantly reducing the injection time and allowing high reproducibility of the infection [7, 8].

Previous work, using the zebrafish as a high-throughput *in vivo* model system for the study of *S. epidermidis* and *M. marinum* infection showed to be successful [7, 8]. This system is able to screen for disease progression via robotic yolk injection of early embryos and using fluorescence readout as a measure for the bacterial load. In agreement with this notion, this setup has been optimized and established a highly efficient high-throughput pipeline with the potential to generate large numbers of homogenously infected embryos and track the progression of the infection during the time after treatment with a number of compounds. With the established setup it is possible to generate up

to 8000 synchronous embryos to screen for disease progression, processing in this way up to 2500 embryos per hour. Embryos are sorted based on their bacterial load using an automated system, ensuring homogenous groups of infected larvae. Furthermore, to validate the setup, effects of reference known to prevent tuberculosis progression in mammals have been tested on embryos infected with *M. marinum* E11 strain or the more virulent M strain [9].

This study describes in detail the high-throughput pipeline that has been established to be able to generate large numbers of infected embryos and the subsequent analysis of the bacterial progression during development and after compound treatment.

Protocol

1) Bacterial strains and growth conditions

1.1) Prepare *S. epidermidis* inoculum

1.1.1) Take several individual colonies from *S. epidermidis* strain O-47, containing a pVW189 derived mCherry expression vector (De Boer L. unpublished) from a Luria Bertani (LB) agar plate supplemented with 10 µg/ml chloramphenicol and culture overnight at 37°C in 25 ml LB medium supplemented with 10 µg/ml chloramphenicol to mid-log stage.

1.1.2) Centrifuge 1 ml of the culture at 12000 × g for 1 min and subsequently wash them 3 times with 1 ml sterile phosphate-buffered saline (PBS) with 0.3% V/V Tween₈₀.

1.1.3) Measure the optical density at 600 nm (OD₆₀₀), and dilute the bacterial suspension to an OD₆₀₀ of 0.3 in 2% W/V polyvinylpyrrolidone₄₀ (pvp₄₀) in PBS. Note: an OD₆₀₀ of 0.3 corresponds to 1.0×10⁸ colony forming unit/ml (cfu/ml).

1.2) Prepare *M. marinum* inoculum

1.2.1) Take several individual colonies from *M. marinum* strain M or E11 containing the pSMT3-mCherry vector stably expressing mCherry [10] from a Middlebrook 7H10 agar plate with 10% V/V Middlebrook OADC enrichment supplemented with 50 µg/ml hygromycin and culture overnight at 28°C in 10 ml of Middlebrook 7H9 broth with 10% V/V Middlebrook ADC enrichment supplemented with 50 µg/ml hygromycin.

1.2.2) Centrifuge 1 ml of the culture at 12000 × g for 1 min and subsequently wash it 3 times with 1 ml sterile PBS with 0.3% V/V Tween₈₀.

1.2.3) Measure the OD₆₀₀, and dilute the bacterial suspension to an OD₆₀₀ of 0.3 in 2% W/V pvp₄₀ in PBS. Note: an OD₆₀₀ of 1 corresponds to 1.0×10⁸ cfu/ml.

2) Prepare zebrafish eggs

2.1) Place maximum 70 male and 50 female wild type zebrafish separately into the large breeding vessel. Note: place the female fish in the lower part of the large breeding vessel.

2.2) Remove the separator the next day in the morning, to let the zebrafish start breeding.

2.3) Collect the eggs at the bottom of the large breeding vessel through the egg collector in a 50 ml tube filled with egg water (60 µg/ml Instant ocean sea salt).

3) Injection needles

3.1) Obtain commercially available custom made glass capillaries needles with an inner diameter of 10 µm.

4) Experimental outline of injection

4.1) Boil 100 ml of 1% W/V agarose in egg water, and cool until approximately 40°C. Pour agarose into the automated micro-injectors plate and place the 1024 well stamp into the agarose. Note: the plate is ready to use when cooled down.

4.2) On the automated micro-injector operating software click on 'Calibrate stage', then click on '1024' well grid and place the agarose plate in the micro-injector and calibrate the plate by clicking on the screen at the centre position of the well.

4.3) Go to 'needle menu' and click on 'calibrate needle holder'.

4.4) Fill the injection needle using a microloader tip with either 10 µl pvp₄₀ containing 100 cfu/nl *S. epidermidis* or 30 cfu/nl *M. marinum*, or use pvp₄₀ as mock injection.

4.5) Place the needle in the automated micro-injector and calibrate the x, y position by lowering or moving the needle up and clicking on the screen at the position of the needle. Then calibrate the z position of the needle by clicking on the screen at the position of the tip of the needle.

4.6) Distribute the eggs over the agarose grid using a plastic transfer pipet, and remove excess egg water. Place the agarose grid in the automated micro-injector.

4.7) Go to the 'Injection menu' and adjust the 'Injection pressure' setting to 200 hPa, 'Injection time' 0.2 s and 'Compensation pressure' 15 hPa, which correlates with 1 nl, at

the Femtojet settings menu.

4.8) Click on 'Inject all' to inject the entire plate.

4.9) Collect the eggs after injection by washing them into a Petri dish (92 × 16 mm), with a maximum of 70 embryos per Petri dish, and incubate at 28°C.

5) Flow-cytometer analysis

5.1) Prepare the large particle flow cytometer according to the manufacturer's instructions and fill the sample cup and sheath fluid container with egg water.

5.2) At the operating software, go to the 'PMT' menu and use the following settings: 650 V for the 'Red' channel and 0V for the 'Green' and 'Yellow' channel. Then go to 'Thresholds' menu and use the following settings: 'Optical density' threshold signal: 975mV (COPAS XL value: 50) and the 'Time Of Flight' (TOF) minimum to 320 μs (COPAS XL value: 800) in order to reduce the influence of debris.

5.3 For analysis without sorting the embryos go to step 5.4, for analysis and sorting the embryos into a Petri dish go to step 5.5 or for analysis and sorting the embryos into a 96 well plate go to step 5.6.

5.4) Place the embryos in the sample cup and click on 'start' to start the analysis. When all embryos are analyzed stop the analysis by clicking on 'stop'. Save the data by clicking on 'Store'. Note: all data is stored as TXT, LMD, DAT, CSV and BSRT files. Follow the protocol at step 5.7.

5.5) Place the embryos in the sample cup and define the maximum of 70 embryos per plate to be sorted by entering 70 in the 'Sort' menu. Place an empty Petri dish under the sorter and click on 'Manual Sort'. When the Petri dish is filled, save the data by clicking on 'Store'. Note: all data is stored as TXT, LMD, DAT, CSV and BSRT files. Follow the protocol at step 5.7.

5.6) Place the embryos in the sample cup and define the maximum of 1 embryo per well to be sorted by entering 1 in the 'Sort' menu. Place an empty 96 well plate into the left plate holder and click on 'Fill plate'. When the 96 well plate is filled, save the data by clicking on 'Store'. Note: all data is stored as TXT, LMD, DAT, CSV and BSRT files. Follow the protocol at step 5.7.

5.7) Get the TXT file to process the raw data, use the following data filter settings: 'Status select': 40, and if using the sort module 'Status sort': 6. Then use the numbers from the total fluorescence signal from the 'Red' channel to calculate the average and the standard error of the mean. Plot these data sets into bar or scatter graphs.

6) Drug treatment

6.1) Analyze and sort at 3 days post injection (dpi), *M. marinum* infected embryos in two equal groups using the large particle flow cytometer (step 5.5). Treat one group with a compound of interest in its carrier solvent and other with carrier solvent alone (control). Apply similar treatments to mock-injected control to test for antibiotic side effects.

6.2) Repeat at 4 and 5 dpi the analysis (step 5.5) and refresh the egg water or egg water containing the compound.

7) High-resolution imaging

7.1) Anesthetize the embryos with 0.02% W/V buffered 3-aminobenzoic acid ethyl ester (Tricaine) in egg water 10 min before analysis.

7.2) Prepare the Vertebrate Automated Screening Technology system and the Large Particle Sampler according to the manufacturer's instructions.

7.3) Select the reference images corresponding to the age of the embryos from the 'Imaging – Object – Detection Setup' menu.

7.4) Select the amount of pictures and orientation to be made by the Vertebrate Automated Screening Technology system from the 'Imaging – Auto store images' menu.

7.5) Place a 96 well plate filled with embryos (from step 5.6) into the left plate holder of the Large Particle Sampler, and click on 'Run plate'.

7.6) When an embryo is detected and correctly positioned; image the head and the tail separately with the CLSM using a 10X plain dry objective and stitch the images afterwards using image processing software.

Representative results

The present results show that the high-throughput pipeline to study *S. epidermidis* and *M. marinum* infection has been successfully established and that may be extended to other infection models. Firstly, the use of the large breeding vessel (Figure 1A), based on the published system by Adatto et al. 2011 [11], enables to generate large numbers of synchronous eggs in single events affording a high control of the spawning process. Next to be able to inject large number of embryos in a short period of time, an improved version of the previously developed automated micro-injection system [7] was used (Figure 1A). To assess which is the best developmental stage for yolk infection, injections

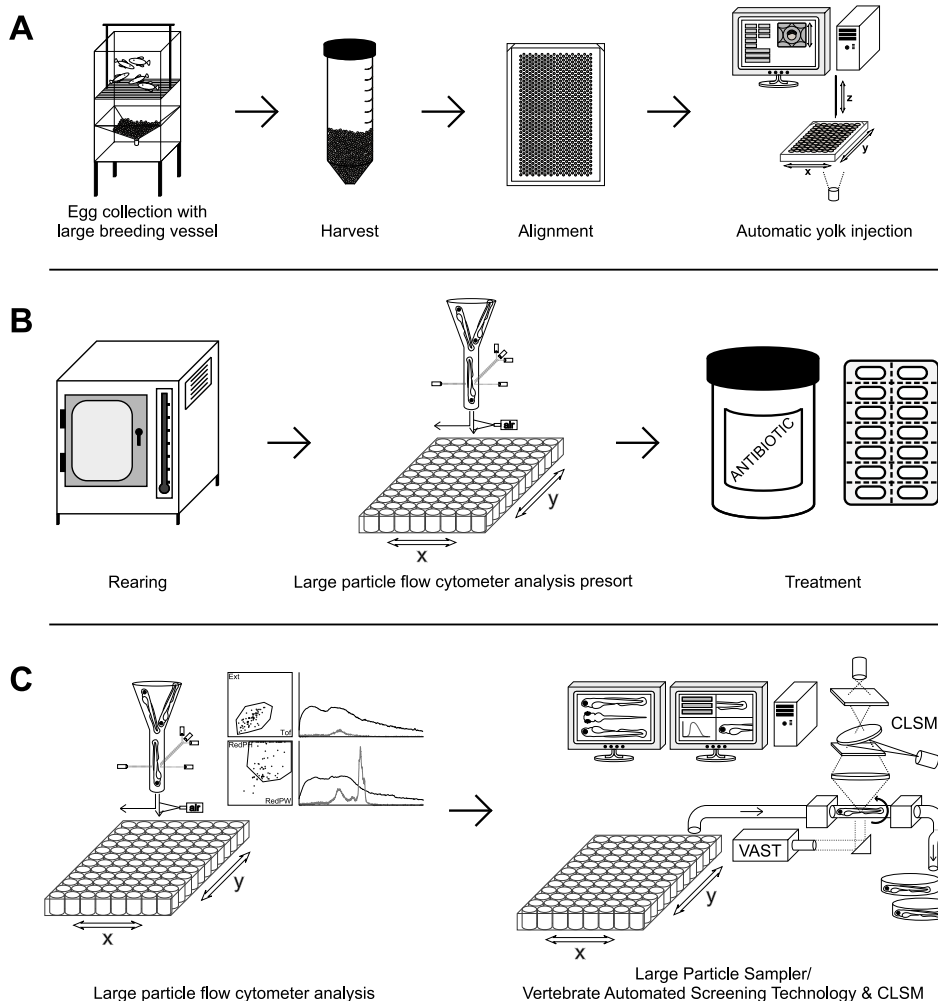


Figure 1: Mainstream experimental outline. (A) Adult fish are put together to mate, eggs are collected, aligned into an agarose plate and injected. (B) The injected eggs are incubated at 28°C and will be pre-sorted for possible drug treatment. (C) Subsequent analysis by large particle flow cytometer and/or Large Particle Sampler/Vertebrate Automated Screening Technology with CLSM.

with *S. epidermidis* and *M. marinum* were performed at all the different stages between 1 and 512 cell stage, according to the description made by Kimmel et al. 1995 [12]. Injections with 100 cfu *S. epidermidis* between the 16 and 128 cell stage provided the best infection pattern (Figure 2). The bacteria proliferated inside the yolk for 3 days and spread into the body from 3 dpi onwards. Performing injections before the 16 cell stage led to high mortality from 4 dpi, and injection after the 256 cell stage showed mainly bacterial growth inside the yolk with hardly any bacteria spreading inside the body of the embryo. Quantification of bacterial burden was performed by fluorescence intensity analysis using the large particle flow cytometer as described by Veneman et al. 2013 [8] (Figure 3).

S. epidermidis O-47

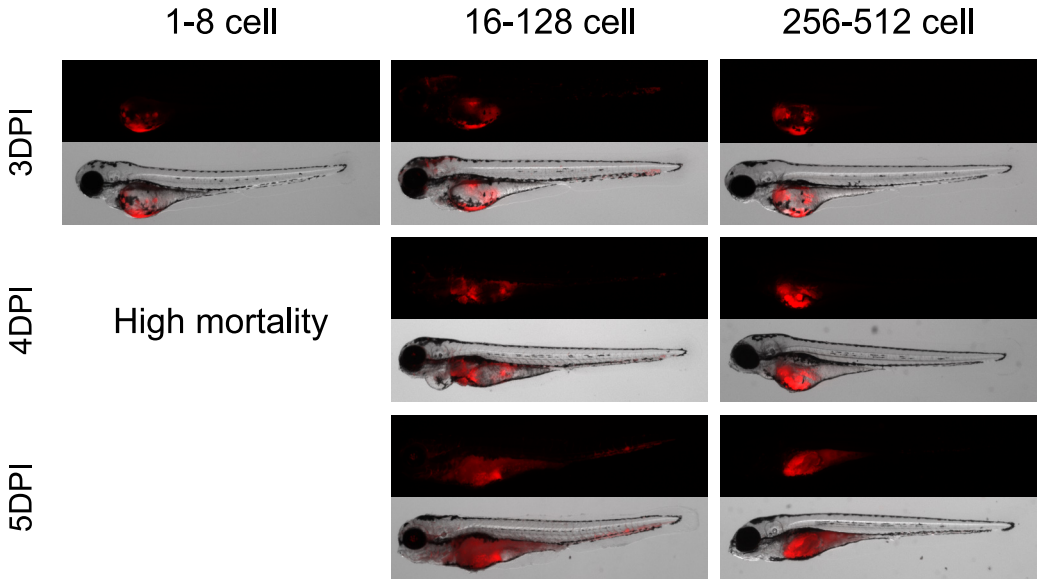


Figure 2: Establishment of best cell stage for *S. epidermidis* yolk injection. Zebrafish embryos were injected in the yolk at different developmental stages from 1 to 512 cell stage with 100 cfu of *S. epidermidis*. Embryos injected between 1 and 8 cell stage showed bacterial growth in the yolk and high mortality from 4 dpi. Embryos injected between 16 and 128 cell stage showed bacterial growth in the yolk and inside the body starting at 3 dpi. Embryos injected between 256 and 512 cell stage showed many bacterial growth inside the yolk.

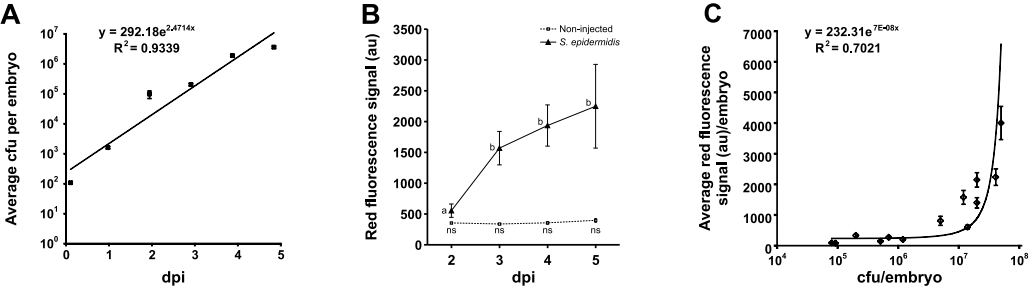
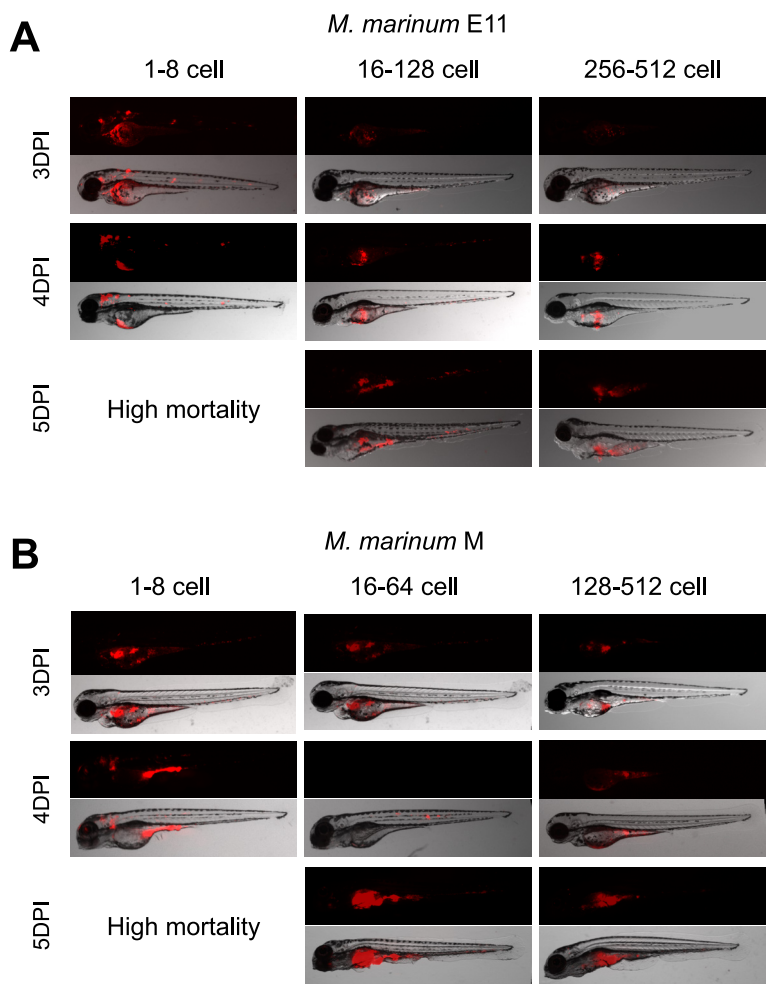


Figure 3: Quantification of bacterial burden using large particle flow cytometer. 100 cfu of *S. epidermidis* were injected into the yolk of zebrafish embryos. **(A)** Up to 5 dpi, each day, groups of 10 embryos were homogenized and plated directly, showing the average exponential growth based on two biological replicas (error bars = SEM). **(B)** Large particle flow cytometer analysis shows the average fluorescence signal from non-injected and *S. epidermidis* injected embryos. 30-160 embryos per condition were analyzed (error bars = SEM), different letters indicate statistical significant differences by one-way ANOVA followed by Tukey's post-hoc test ($P < 0.001$), ns: not significant differences. **(C)** Correlation between cfu and average fluorescence signal of groups of 10 *S. epidermidis* infected embryos (error bars = SEM). This figure has been modified from Veneman et al. 2013 [8].

Observations showed that the optimal developmental stage for injection of 30 cfu *M. marinum* injection is between 16 to 128 cell stage for the E11 strain (Figure 4A) and between 16-64 cell stage with the more virulent M strain (Figure 4B). Embryos injected at these stages showed bacterial growth inside the yolk and spreading of the bacteria through the embryo (Figure 7). Infection with both strains at earlier stages presented non-specific generalized bacterial growth leading the embryos to die after 4 dpi. On the other hand, in embryos injected at later stages bacterial burden was restricted to the yolk.



Next, pre-sorting with large particle flow cytometer (Figure 1B) generated large homogenous groups of infected fish excluding non- or highly infected embryos (Figure 5A and 6A). After pre-sorting, *M. marinum* infected embryos were treated with Rifampicin, a first-line anti-tuberculosis drug. Previous studies demonstrated that treatment with Rifampicin at a dose of 200 μ M efficiently reduces *M. marinum* infection in zebrafish [7, 13]. Taking advantage of the large number of homogeneously infected embryos generated with the high-throughput setup, treatment with different doses was performed. Embryos infected with *M. marinum* M strain and treated for 48 hours with 12, 24 and 200 μ M Rifampicin showed to reduce efficiently mycobacterial infection in a dose dependent manner (Figure 5B). In view of the efficient reduction of the infection using Rifampicin at a dose of 200 μ M this concentration was used for the future experiments. In line with the previous result, studying bacterial burden progression using *M. marinum* E11 strain a significant reduction 24 hours and onwards after treatment with 200 μ M Rifampicin was observed (Figure 6B).

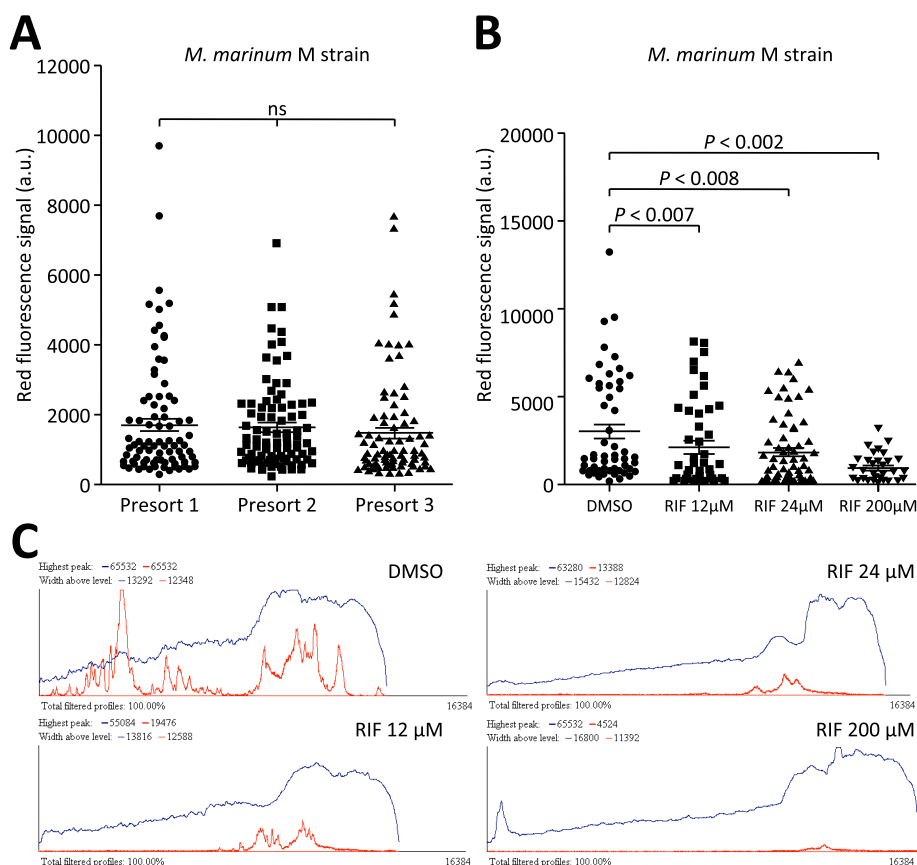


Figure 5: Treatment of *M. marinum* acute infection with a first-line anti-tuberculosis drug. Embryos injected between 16-64 cell stage with 30 cfu of *M. marinum* M strain were run through the large particle flow cytometer at 3 dpi to be sorted in two groups after discarding the non- and/or highly infected embryos. (A) Fluorescence of individual embryos in both groups. (B) Embryos treated with Rifampicin (RIF) for 48

hours at doses of 12, 24 and 200 μM were analyzed at 4 dpi; their bacterial load is significantly reduced. (C) Representative COPAS profiles of embryos treated with DMSO and Rifampicin at doses of 12, 24 and 200 μM for 24 hours. Bacterial load and distribution is indicated by the red peaks. Blue line represents the profile of the element sorted (4 dpf zebrafish embryo) by the COPAS. 60-90 embryos per condition were analyzed. Each data point represents an individual embryo. Values are indicated as mean $\pm$ SEM. ns: not significant differences. Analysis of statistical significance of differences was performed by one-way ANOVA followed by Tukey's post-hoc test.

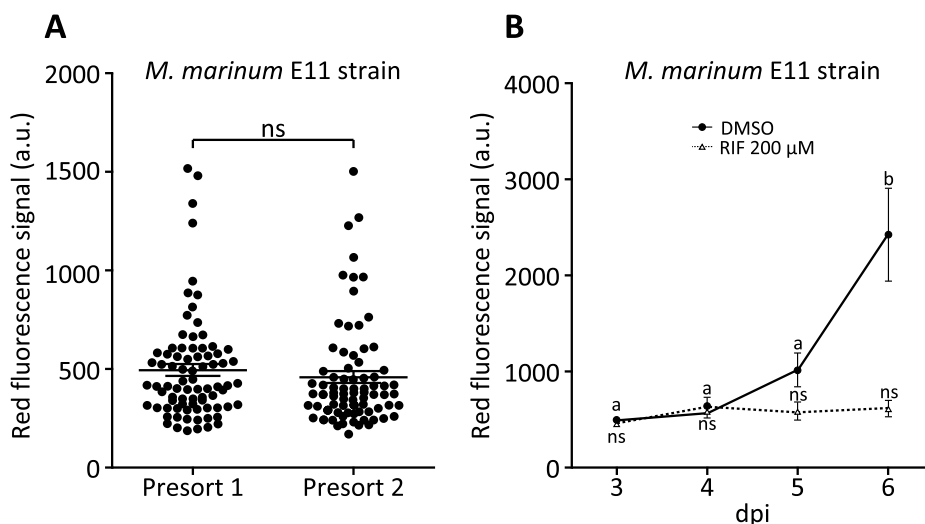


Figure 6: Treatment of *M. marinum* chronic infection with a first-line anti-tuberculosis drug. Embryos injected between 16-64 cell stage with 30 cfu of *M. marinum* E11 strain were run through the large particle flow cytometer at 3 dpi to be sorted in two groups after discarding the non- and/or highly infected embryos. (A) Fluorescence of individual embryos in both groups. (B) Embryos treated with Rifampicin (RIF) at 200 μM during 4 days were analyzed showing a significant reduction of the bacterial load after 1 day of treatment. 90 embryos per condition were analyzed. Values are indicated as mean $\pm$ SEM. Different letters indicate significant differences between time points of the same treatment. * indicates significant differences with control group. ns: not significant differences. Analysis of statistical significance of differences was performed by one-way ANOVA followed by Tukey's post-hoc test. ($P < 0.05$). Figure B) has been modified from Spaink et al. 2013 [13].

Furthermore, if high magnification imaging is required of these embryos, they can be displayed automatically in 96 well plates (Figure 1C), from where the samples can be analyzed using the Vertebrate Automated Screening Technology system with the Large Particle Sampler mounted onto a CLSM. The Vertebrate Automated Screening Technology system with the Large Particle Sampler is a system that can either be mounted onto a CLSM or stereo microscope. This device allows the loading of live or fixed embryos from a 96 well plate or bulk container automatically through a glass capillary, and orientates it in front of the camera at the desired angle (e.g. dorsal or lateral). Images of the embryo in all orientations can be made with the on board camera or with an external CLSM (Figure 7). Embryos will subsequently be transferred in the collection or waste container.

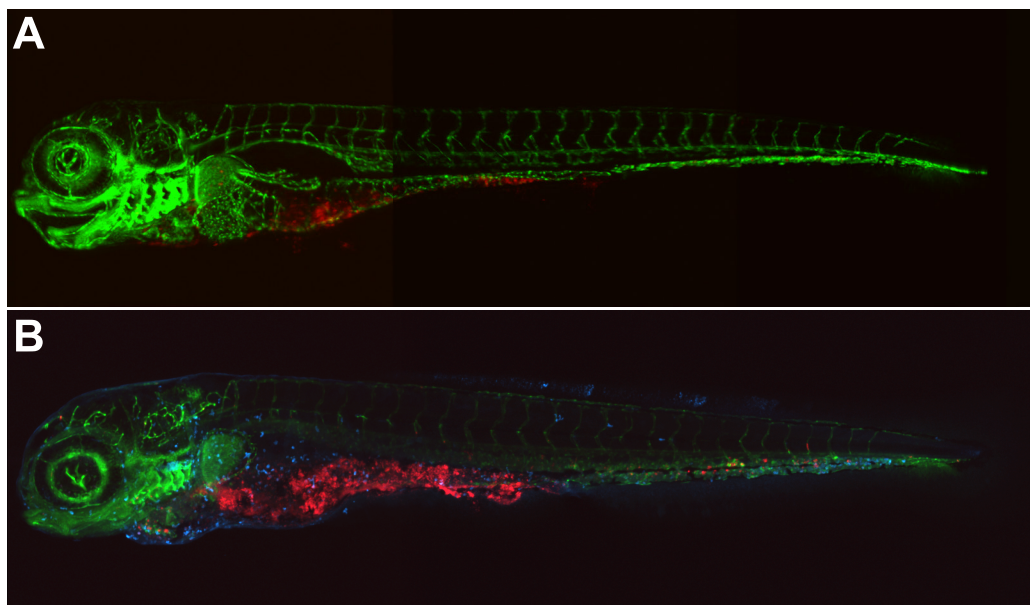


Figure 7: Result of *M. marinum* E11 yolk injection imaged using Vertebrate Automated Screening Technology and CLSM. Confocal Z stack (stitched 3 images) of a 5 dpi *Tg(fli1-egfp)* [17] embryo. (A) Live embryo showing proliferation of *M. marinum* E11 bacteria (red) throughout the body. (B) Fixed 5 dpi *Tg(fli1-egfp)* embryo showing *M. marinum* E11 bacteria (red) throughout the body co-localizing with leucocytes (light blue) detected by L-plastin immunostaining [18].

Discussion

The high-throughput methodology described in this paper provides a fast and cost effective pipeline to screen high number of fish embryos and larvae with different types of infections. Using the large breeding vessel instead of traditional single or family breeding tanks facilitated control of the spawning process and generation of larger number of synchronous eggs. With an improved version of the automated micro-injection system [7], it is possible to inject up to 2500 eggs almost all in the same cell stage within 1 hour. With these updates and improved software it is feasible to inject more eggs than previously was possible which can be used to perform large drug screens with bacterial proliferation as a read out. However this method is still limited to yolk injection, other injection routes for example described by Benard et al. 2012. [6], will hopefully be incorporated in the automated micro-injection system in the near future.

Although these methods are benchmarked for screening zebrafish, it would be useful for applications with other fish species as well. For instance, the common carp has been indicated to have advantages for drug screens. Like zebrafish, eggs and early stage embryos from common carp are transparent but with the main advantage of its large spawn size of hundred thousands of eggs and the availability of inbred lines that offer a more constant genetic background [14].

The analysis of large amounts of infected embryos is done with the high-throughput large particle flow cytometer. This device can sort analyzed embryos into multi well plates or a Petri dish making it especially suitable for testing large numbers of compounds. If a higher imaging resolution is needed, than the setup is adapted in a way that the large particle flow cytometer technology can be used for pre-screening and subsequently analyze the samples at a medium throughput at a higher resolution. This can be done using the Vertebrate Automated Screening Technology [15, 16]. This device can automatically collect live or fixed embryos between 2 and 7 days post fertilization from a multi well plate or bulk container, image 360° through a capillary using CLSM or stereo microscopy and dispose again in 2 bulk containers allowing manual sorting of the embryos based on the microscopic images. Future improvements will allow the sorting of the embryo after imaging into the multi well plate, therefore making it possible to screen automatically large number of individual embryos over time with CLSM. Assuming that in future applications the Vertebrate Automated Screening Technology system can also be connected to the large particle flow cytometer technology without the need of prior dispensing larvae into multi well plates, will lead to a more advanced sorting.

This paper describes the establishment and optimization of a high-throughput setup to study *S. epidermidis* and *M. marinum* infection as a model for drug discovery. It demonstrates that the outcome of these bacteria injected into the yolk depends on the developmental stage of the eggs at the time of injection. Injecting *M. marinum* E11 at 16-128 cell stage or the M strain at 16-64 cell stage leads to the same infection pattern as caudal vein injection [2, 6]. However this setup is not limited to the proliferation of bacterial pathogens only. It was shown before that it is possible to robotically inject solutions containing DNA, RNA or morpholinos for transgenesis, over-expression and gene knock-down studies, respectively [13]. Furthermore, it was shown that this setup is also useful for the study of cancer cell proliferation and migration. Therefore this pipeline presents a versatile method for high-throughput screens of a variety of signal mechanisms in the context of innate immunity, applied to infectious disease and the development of cancer. These screens can be combined with others for medicine discovery but also with analysis of possible toxic effects of identified applicable drugs.

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Supplementary material

Supplementary material can be found on:

<http://www.jove.com/video/51649/establishment-optimization-high-throughput-setup-to-study>



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