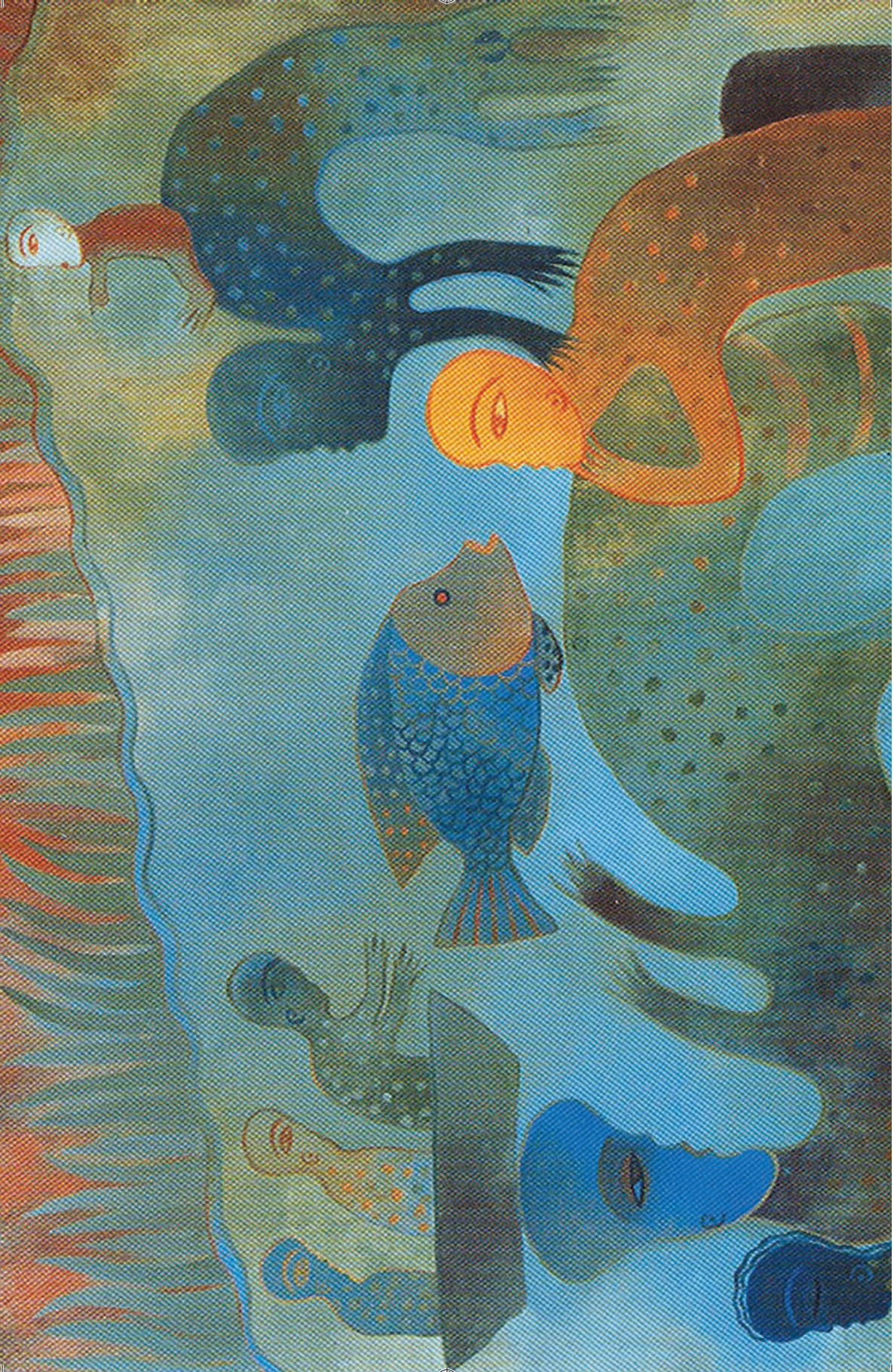

CHAPTER 4.2

NEW HIGH-THROUGHPUT TECHNOLOGIES FOR DRUG SCREENING IN ANIMAL MODELS OF TB

Herman P. Spaink, Tom H.M. Ottenhoff, Ron P. Dirks, Annemarie H. Meijer

Rapid screening methods could accelerate the achivement of new vaccines and therapies.

'Waking up is a parachute jump from dreams
free of the suffocating turbulence
the traveler sinks toward the green zone of morning.'

Prelude
Tomas Tranströmer

Conversation with The Fish
Manuel Mendive
Acrilyc on canvas; 107.5 × 113.75 cm

ABSTRACT

Approximately one-third of the human population is infected with MTB, the causative agent of TB. The emergence of drug- and multidrug-resistant TB is caused by mycobacterial strains that exhibit antibiotic resistance to the current standard treatments. The discovery of new compounds and targets against both drug- and multidrug-resistant TB strains of MTB is therefore crucial. However, the lack of high-throughput animal models that adequately represent the different phases of human disease has severely hampered progress in this field (1, 2). To overcome this obstacle, we have made an attempt by using zebrafish, a host organism to *Mycobacterium marinum,* as our model system. *M. marinum* is very closely related to the human pathogen MTB, and is the causative agent of TB in fish. Importantly, research has shown that hallmarks of human TB, most notably the process of granuloma formation, are remarkably similar between fish and humans. Zebrafish produce large clutches of eggs and their embryos develop rapidly. The initial stages of granuloma formation in zebrafish embryos can be excellently visualized due to their external development and optical transparency, thus making it a valuable vertebrate model amenable to high-throughput research. We have recently developed an automatic injector that can reliably infect up to 2,000 early-stage embryos per hour. Having validated our system using *M. marinum*, we subsequently demonstrated that our automated approach allows high-throughput infection of zebrafish with MTB. In addition, we have proven that the human pathogen survives and spreads through immune cells in this surrogate host (3). Moreover, the established MTB infection could be treated and cured with first-line anti-TB drugs, indicating the translational power of this model. Thus, we have successfully developed an automated high-throughput system capable of infecting zebrafish embryos with *M. marinum* and MTB thus making it suited for testing novel anti-TB compounds *in vivo*.

The alarming rate of the emergence of new drug resistant (MDR/XDR) strains of MTB isolated from patients, in particular HIV-infected individuals, is reason for global concern. This, in turn, has spurred development of new, effective vaccines,

as well as discovery of novel antibiotics targeting either the pathogen or the host (2, 4, 5). While *in vitro* models have shed light on processes that are central to the uptake and survival of the bacteria within the macrophages, they cannot represent the exact phenotype of MTB infection observed *in vivo*. MTB can persist for many years in specialized structures of infected and non-infected immune cells known as granulomas, which are extremely difficult to recapitulate in an *in vitro* model (6). Due to the persistence of MTB in granulomas and its capability to subvert the effects of treatment by developing drug tolerance, long-term multidrug therapy is required in TB treatment to overcome tolerance. The complex interactions between MTB or other pathogens with their hosts could explain the discrepancies on drug efficacy observed between *in vitro* and *in vivo*. Therefore, *in vivo* animal models are essential for novel drug development to combat bacterial infections in humans. The use of non-human primate models that exhibit similar hallmarks of the human TB infection has been conducted in previous studies (1, 7). Using the murine model of TB, which offers extensive arrays of immunological reagents and genetic tools has strongly contributed to the understanding of the disease process in humans with TB infection. On the other hand, the mouse model of TB has shown to form loosely structured granulomas without a caseating necrosis center that do not resemble the well-structured and centrally necrotizing granulomas in humans (2, 8). The guinea pig model has the advantage of displaying a well-structured granuloma formation and has therefore been used to validate anti-TB vaccines and drugs (9). Like guinea pigs, rabbits also display a disease spectrum that resembles many of the specific stages of human disease, including the formation of caseating granulomas (1, 8, 10). Recently, a new model of TB was developed in mini-pigs that could provide a better understanding in TB infection control (11). Following a transthoracic infection involving a low dose of MTB, the encapsulated granuloma structures in the lungs of the mini-pigs were found to be similar to TB granulomas in humans. The model was further tested in isoniazid chemotherapy combined with MTB fragment-based immunotherapeutic vaccination. While guinea pigs, rabbits or mini-pigs offer useful alternatives to murine models, these models are very costly and cannot be used in a high-throughput setting.

Use of zebrafish as an alternative vertebrate animal model for TB research has gained much attention in recent years based on its many promising characteristics that enable a rapid high-throughput drug screening for immune-related diseases, including inflammatory and infectious diseases and cancer (12). This is due to the excellent possibilities for *in vivo* imaging in combination with advanced tools for genomic and large scale mutant analysis. The context of the embryo's developing immune system makes it possible to study the contribution of different immune cell

types to disease progression. Furthermore, due to the clear temporal separation of innate immunity from adaptive responses, zebrafish embryos and larvae are particularly useful for dissecting the innate host factors involved in pathology (13). Recent studies have underscored the remarkable similarity of the zebrafish and human immune systems, which is important for biomedical applications. Zebrafish are also amenable to forward genetic screening, or reverse genetics techniques such as injection of morpholinos (synthetic oligonucleotides inhibitory of mRNA translation or pre-mRNA splicing) (14, 15).

As an ectotherm, zebrafish is one of the natural hosts of *M. marinum*, a close relative of the MTBC (16) which can cause localized granulomatous skin infection in the distal parts of the extremities in humans (17). Of crucial relevance, as shown by the pioneering work of the Ramakrishnan group, *M. marinum* infection of zebrafish closely mimics the mammalian TB pattern of infection with a rapid increase in bacterial numbers during the initial stage of infection, and the formation of caseous granulomas which present characteristics typical of their human counterparts (18–21).

The indirect study of human TB via the infection of the zebrafish embryo with *M. marinum* has already led to the clarification of many important processes in the life cycle of the infection, in particular those underlying the mechanisms of granuloma formation (20–25). Infection of zebrafish embryos demonstrated that granuloma formation is initiated in the sole context of innate immunity, prior to the development of T-cells (21). At later stages of zebrafish development where the adaptive immune system starts to develop (i.e. approximately 3 week post fertilization), the resulting granulomas are highly similar to the granulomas found in human tissues (26). The importance of studying mycobacterial infections at the whole organism level was highlighted in the report that induction of *mmp9* expression, enhancing macrophage recruitment to granulomas, was localized to epithelial cells near infected macrophages (24). Another example of the use of zebrafish larvae to uncover a host-pathogen interaction relevant to human mycobacterial infection is the recent forward genetic screen by Tobin and Ramakrishnan, who mapped a hypersusceptibility mutation to the leukotriene biosynthesis gene, *lta4h*, and showed that heterozygosity at the *LTA4H* locus correlated with susceptibility of human populations to both TB and leprosy (27). *M. marinum*-infected zebrafish larvae were recently deployed for *in vivo* characterization of anti-TB drug activity and drug-tolerance (28). Results of this study demonstrated that multidrug-tolerant bacteria arise within days of infection, are enriched among the replicating intracellular population, and are amplified and disseminated by the tuberculous granuloma. Drug tolerance was reduced by application of bacterial efflux pump

inhibitors such as verapamil. The zebrafish embryo model has also proven to be useful to screen an *M. marinum* transposon mutant library for novel virulence mutants affected in granuloma formation (29). It is therefore clear that the zebrafish mycobacterial infection model is an attractive and advantageous alternative for analyzing granuloma formation, disease progression, and infection control *in vivo*.

The common route of infecting zebrafish embryos with *M. marinum* is the injection of the pathogen into the caudal vein of the 1 day old embryo (21). This method is labour-intensive and generally considered to be a low-throughput technique, leading to major bottlenecks in drug discovery particularly in times of high-throughput technology and large chemical library availability. Since infection by immersion is not an effective alternative, we sought to establish a reliable high-throughput automatic injection system, which could drastically reduce labor intensity while at the same time also vastly increase the number of reproducibly infected embryos (3). Large quantities of similarly-injected/infected embryos would then allow the testing of sizeable compound libraries for anti-bacterial activity targeting either the pathogen or the host itself (5). To develop such an automated injection system, we first demonstrated that the injection of 20–40 *M. marinum* colony-forming units (CFUs) into the yolk sac of embryos at early developmental stages (from the single cell up to the 1,024-cell stage) mimics the infection obtained with the well-established caudal vein injection method (Figure 4.2.1). Subsequently, we developed a robotised system around the yolk injection concept (Figure 4.2.2). The injector robot produced identical results to manual yolk injections. During injections, eggs are held in an agarose grid, which allows highly reproducible alignment, such that the yolk injections do not require camera control. With this set-up 2000 injections were achieved per hour with a success rate of over 99%. The use of polyvinylpyrrolidone as a polymer-based carrier for the fluorescently-labelled bacteria improved concentration homogeneity and visibility of the injected inoculum. The injector can be coupled to a flow cytometer capable of fluorescence-based sorting of live multicellular organisms (Complex Object Parametric Analyzer and Sorter, COPAS). The first application of the COPAS system is within hours after injection and serves to sort injected eggs into multi-well plates, simultaneously discarding unfertilized or uninjected eggs (Figure 4.2.3A). The second application of the COPAS system is the analysis of infection in the developing larvae at 4 to 5 days post injection. The COPAS readout provides the total level of fluorescence, representative of bacterial load, and a distribution profile of fluorescence over the larval body, which allows convenient assessment of granuloma spreading (Figure 4.2.3B). Demonstrating the applicability to drug screens, COPAS analysis was shown to discriminate correctly between untreated larvae and larvae treated with first-line

anti-TB drugs (rifampicin and isoniazid). Thus, the automatic injector, coupled with COPAS sorting, provides a powerful high-throughput pipeline for infecting and analyzing zebrafish embryos and offers a new *in vivo* tool for rapidly testing the efficacy of large panels of molecules on the propagation of the pathogen studied.

Practical advantages of using *M. marinum* for high-throughput drug screening are that it can be used at lower biosafety level (BSL-2) than MTB (BSL-3) and has a faster growth rate. Nevertheless it was important to show that the zebrafish embryo model can also be used to study proliferation and tissue spreading of the human pathogen, MTB. The use of the injector robot has been instrumental to overcome technical difficulties of injecting a BSL-3 pathogen. Its application enabled a high biosafety-level study of MTB infection of zebrafish embryos, showing that this pathogen can persist in macrophages during zebrafish larval development and was sensitive to combinatorial rifampicin and isoniazid treatment in this model similar to *M. marinum* (3). Interestingly, we have recently demonstrated the applicability of this robotic injection system for the study of other bacterial infections, such as streptococcal species that are often associated with problems with prosthetic implants (unpublished results). We expect that we can also investigate the role of antibacterial factors in such screenings, for instance the effect of co-injected antibodies. Furthermore, genetic tools to investigate the host immune system, such as antisense morpholinos, can be easily integrated into our set-up. This shows its general relevance in the high-throughput study of diseases that benefit from the use of whole vertebrate organisms.

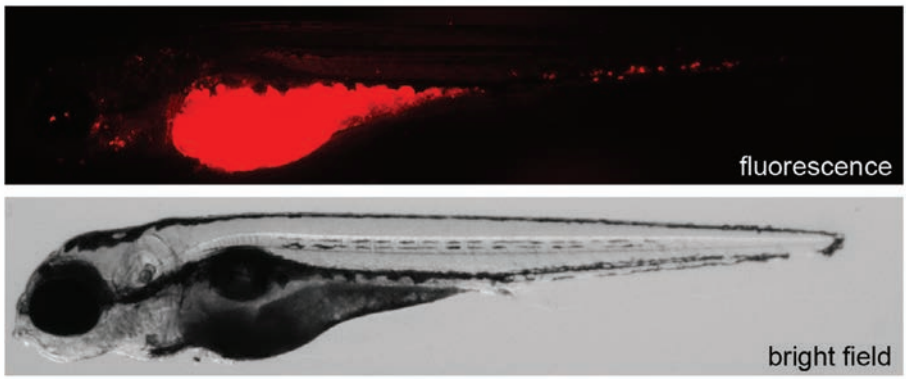

Figure 4.2.1 Zebrafish larva at 5 days after injection of mCherry-labeled Mycobacterium marinum bacteria into the yolk. Spreading of bacteria into granuloma-like aggregates can be observed in the head and tail area

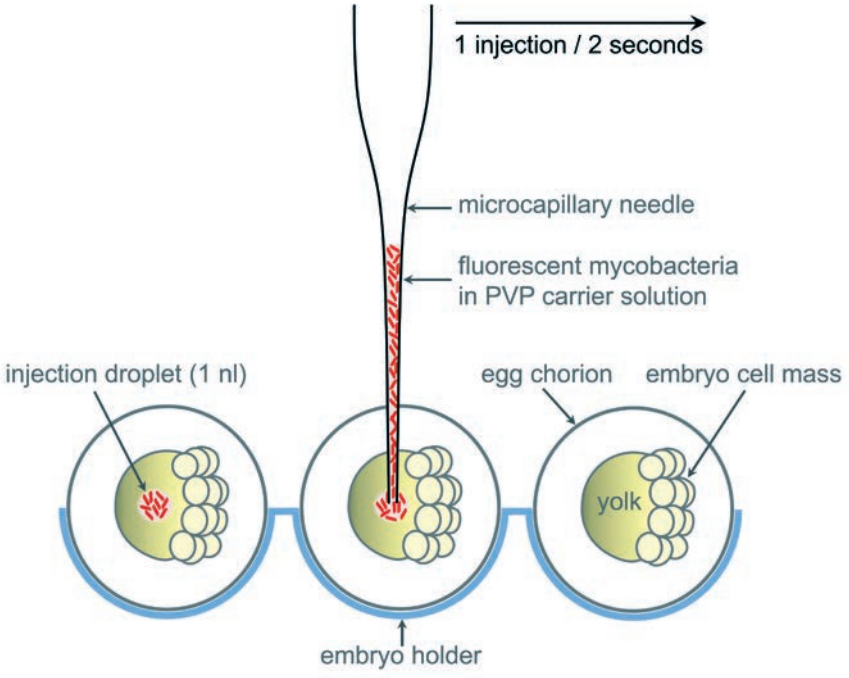

Figure 4.2.2 Schematic presentation of high-throughput injection of zebrafish embryos with mycobacteria. Further details and movies are given in Carvalho *et al*. (3)

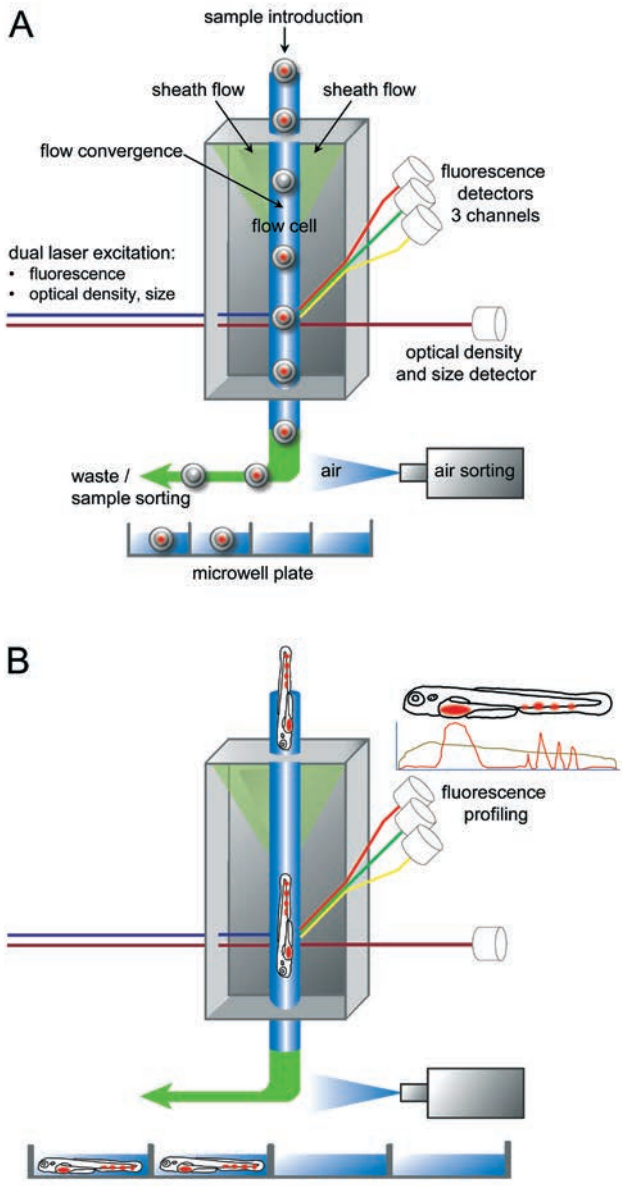

Figure 4.2.3 High-throughput screening of mycobacterium infection using the COPAS system. (A) Sorting of infected embryos immediately after yolk injection. (B) Fluorescence profiling of mycobacterial spreading into granuloma-like aggregates in larvae at 5 days post injection (flow cell scheme courtesy of Union Biometrica)

CONCLUSIONS

Carvalho *et al*. (3) showed for the first time that MTB can be used to infect zebrafish embryos, and replicate a hallmark feature of TB in this *in vivo* model, namely persistent infection of macrophages which are parasitized by the bacterium. The zebrafish had already been shown to be an excellent vertebrate model for the study of granuloma formation and progression when infected with the fish pathogen *M. marinum* (24, 27). The possibility of direct infection of zebrafish with MTB was originally viewed with scepticism, partly due to the absence of lungs in this organism; it is, however, important to keep in mind that human TB infection is a systemic disease of the entire body, and this disease can manifest itself in many extrapulmonary sites. We presented proof that the zebrafish is a host for MTB, and that infection of zebrafish embryos with this human pathogen leads to symptoms similar to those seen in *M. marinum* infections. We can infect zebrafish embryos using a novel robotic screening system that operates at an unprecedented high-throughput level, and demonstrated that it can be used to screen for TB disease progression in an entire living vertebrate. This animal model presents a significant new tool in the study of many types of disease for which no other good cellular models are available. We further show that the result of first-line drug treatment of TB in zebrafish reliably mimics the treatment of human patients. Future research will need to investigate later stages of disease progression. This will make it possible to investigate the role of the adaptive immune system in response to MTB infection in zebrafish. If, as expected by earlier studies with *M. marinum* infection, this also leads to the development of caseous granulomas in adult tissue and we can expect that zebrafish will also contribute to screening for T-cell and antibody responses. It is therefore not improbable that high throughput screening approaches in the zebrafish will facilitate vaccine development. This approach may also facilitate the screening of *M. marinum* (and MTB) mutants that might impact on developing novel live vaccine strains.

It should be noted that the availability of many additional fish models will also be important for future research. For example, the common carp is a closely related fish species with large body weight compared to zebrafish and can produce hundred thousands of genetically homogeneous embryos. As carp embryos can also be infected with *M. marinum*, this model has great potential to facilitate drug screening and further increase the throughput level.

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REFERENCES

1. Young D. Animal models of tuberculosis. *Eur J Immunol*, 2009; 39: 2011–4.

2. Ottenhoff TH. Overcoming the global crisis: "Yes, we can", but also for TB … ? *Eur J Immunol*, 2009; 39: 2014–20.

3. Carvalho R, de Sonneville J, Stockhammer OW, Savage ND, Veneman WJ, Ottenhoff TH, et al. A high-throughput screen for tuberculosis progression. *PLoS One*, 2011; 6: e16779.

4. Russell DG, Barry 3rd CE, and Flynn JL. Tuberculosis: what we don't know can, and does, hurt us. *Science*, 2010; 328: 852–6.

5. Kuijl C, Savage ND, Marsman M, Tuin AW, Janssen L, Egan DA, et al. Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature*, 2007; 450: 725–30.

6. Russell DG. Who puts the tubercle in tuberculosis? *Nat Rev Microbiol*, 2007; 5: 39–47.

7. Flynn JL. Lessons from experimental Mycobacterium tuberculosis infections. *Microbes Infect*, 2006; 8: 1179–88.

8. O'Toole R. Experimental models used to study human tuberculosis. *Adv Appl Microbiol*, 2010; 71: 75–89.

9. Williams A, Hall Y, and Orme IM. Evaluation of new vaccines for tuberculosis in the guinea pig model. *Tuberculosis* (Edinb), 2009; 89: 389–97

10. Gupta UD and Katoch VM. Animal models of tuberculosis for vaccine development. *Indian J Med Res*, 2009; 129: 11–8.

11. Gil O, Díaz I, Vilaplana C, Tapia G, Díaz J, Fort M, et al. Granuloma encapsulation is a key factor for containing tuberculosis infection in minipigs. *PLoS One*, 2010; 5: e10030.

12. Mione M, Meijer AH, Snaar-Jagalska BE, Spaink HP and Trede NS. Disease modeling in zebrafish: cancer and immune responses--a report on a workshop held in Spoleto, Italy, July 20-22, 2009. *Zebrafish*, 2009; 6: 445–51.

13. Meijer AH and Spaink HP. Host-pathogen interactions made transparent with the zebrafish model. *Curr Drug Targets*, 2011; 12: 1000–17.

14. Amsterdam A and Hopkins N. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet*, 2006; 22: 473–8.

15. Nasevicius A and Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet*, 2000; 26: 216–20.

16. Tobin DM and Ramakrishnan L. Comparative pathogenesis of Mycobacterium marinum and Mycobacterium tuberculosis. *Cell Microbiol*, 2008; 10: 1027–39.

17. Arend SM, van Soolingen D, and Ottenhoff TH. Diagnosis and treatment of lung infection with nontuberculous mycobacteria. *Curr Opin Pulm Med*, 2009; 15: 201–8.

18. Swaim LE, Connolly LE, Volkman HE, Humbert O, Born DE, and Ramakrishnan L. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect Immun*, 2006; 74: 6108–17.

19. Volkman HE, Clay H, Beery D, Chang JC, Sherman DR, and Ramakrishnan L. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *PLoS Biol*, 2004; 2: e367.

20. Davis JM and Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell*, 2009; 136: 37–49.

21. Davis JM, Clay H, Lewis JL, Ghori N, Herbomel P, and Ramakrishnan L. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity*, 2002; 17: 693–702.

22. Agarwal N and Bishai WR. Microbiology. Subversion from the sidelines. *Science*, 2010; 327: 417–8.

23. Clay H, Davis JM, Beery D, Huttenlocher A, Lyons SE, and Ramakrishnan L. Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. *Cell Host Microbe*, 2007; 2: 29–39.

24. Volkman HE, Pozos TC, Zheng J, Davis JM, Rawls JF, and Ramakrishnan L. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. *Science*, 2010; 327: 466–9.

25. Clay H, Volkman HE, and Ramakrishnan L. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity*, 2008; 29: 283–294.

26. Lesley R and Ramakrishnan L. Insights into early mycobacterial pathogenesis from the zebrafish. *Curr Opin Microbiol*, 2008; 11: 277–83.

27. Tobin DM, Vary Jr. JC, Ray JP, Walsh GS, Dunstan SJ, Bang ND, et al. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. *Cell*, 2010; 140: 717–730.

28. Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, et al. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell, 2011; 145: 39–53.

29. Stoop EJ, Schipper T, Huber SK, Nezhinsky AE, Verbeek FJ, Gurcha SS, et al. Zebrafish embryo screen for mycobacterial genes involved in the initiation of granuloma formation reveals a newly identified ESX-1 component. *Dis Model Mech*, 2011; 4: 526–36.