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Robotic injection of zebrafish embryos for high-throughput screening in disease models ☆

Herman P. Spaink [a,*], Chao Cui [b], Malgorzata I. Wiweger [a,b], Hans J. Jansen [b], Wouter J. Veneman [a], Rubén Marín-Juez [b], Jan de Sonneville [c], Anita Ordas [a], Vincenzo Torraca [a], Wietske van der Ent [a], William P. Leenders [d], Annemarie H. Meijer [a], B. Ewa Snaar-Jagalska [a], Ron P. Dirks [b]

[a] Department of Molecular Cell Biology, Institute of Biology, Leiden University, The Netherlands
[b] ZF-screens B.V., Leiden, The Netherlands
[c] Life Science Methods B.V., Leiden, The Netherlands
[d] Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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ABSTRACT

The increasing use of zebrafish larvae for biomedical research applications is resulting in versatile models for a variety of human diseases. These models exploit the optical transparency of zebrafish larvae and the availability of a large genetic tool box. Here we present detailed protocols for the robotic injection of zebrafish embryos at very high accuracy with a speed of up to 2000 embryos per hour. These protocols are benchmarked for several applications: (1) the injection of DNA for obtaining transgenic animals, (2) the injection of antisense morpholinos that can be used for gene knock-down, (3) the injection of microbes for studying infectious disease, and (4) the injection of human cancer cells as a model for tumor progression. We show examples of how the injected embryos can be screened at high-throughput level using fluorescence analysis. Our methods open up new avenues for the use of zebrafish larvae for large compound screens in the search for new medicines.

1. Introduction

The use of zebrafish as an animal model has an abundance of applications in fundamental research in vertebrate development, physiology and toxicology [4,11,37,78,79]. More recently, this model has also been shown to be highly applicable for studies of many types of disease [1,2,5,9,35,44,52,55,57,59–61,73–75]. The benefits of the relatively small and transparent larvae for optical imaging using transgenic fish lines expressing many colour varieties of the GFP protein have been widely exploited in these disease studies [1]. Currently the genetic tool box is comprised of a large variety of gene knock-down or knock-out systems [6,10,14,20,21,25,43,64]. Additionally many genomic-based techniques such as RNA deep sequencing, metabolomics and proteomics have been applied to zebrafish [17–19,40,42,45,53,54,66,71,97]. A comparison of parallel deep RNA sequencing and proteome analysis has been reported (Palmblad et al., submitted).

The fact that the innate immune system of zebrafish is highly similar to that of mammals and is already fully functional as early as two days after fertilization makes zebrafish larvae extremely useful for studies of diseases related to the immune system [72,94]. Examples given below are studies of cancer progression and infectious diseases caused by many bacteria, fungi or viruses.

1.1. Zebrafish microinjection and screening tools

Microinjection of zebrafish embryos is an essential technology for the following applications:

- The generation of transgenic zebrafish lines.
- The generation of gene knock-out lines using zinc fingers or TALEN technology.
- Gene knock-down using morpholinos, siRNA or antibodies.
- Overexpression of genes by injection of mRNA.
- The injection of tracer dyes or particles.
- Intraorganismal introduction of microbes in embryos or larvae for infection studies.
- Transplantation of cells between embryos.
- Xenograft implantation of cells for cancer studies.

* Corresponding author. Address: Einsteinweg 55, 2333 CC Leiden, The Netherlands.
E-mail address: h.p.spaink@biology.leidenuniv.nl (H.P. Spaink).

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Many general methods for these applications can be found in the book: Essential Zebrafish Methods: Cell and Developmental Biology [26]. More specifically for injection methods that can be used for these applications we can refer to detailed descriptions in four methodology compendia or books: (1) Zebrafish: Methods and Protocols [51], (2) The zebrafish book, A guide for the laboratory use of zebrafish (*Danio rerio*), 5th edition [99] and (3) Methods in Cell Biology issues 104 and 105 [27,28], which provide comprehensive laboratory protocols and reviews for recent zebrafish methods related to disease models and chemical screens. Recently also video enhanced protocols for zebrafish microinjection have been published, for instance by Benard et al. [7], describing in detail the application of microinjection of bacterial pathogens. In brief, all methods use thin glass capillary needles to introduce compounds or biological materials inside various parts of embryos and larvae. In the embryonic and early larval stages the transparency and softness of the tissues warrants a high success rate of the injection protocol. In contrast, injection in the later larval stages is more difficult due to higher rigidity of tissue and can currently only be performed at relatively low throughput. For the first four applications mentioned above, DNA, RNA or morpholinos can be injected into the yolk of early stage embryos. Due to the relatively large size of the yolk this offers a fast procedure for microinjection that even can be completely automated. Robotic injection of zebrafish embryos using image recognition has been shown to accurately deliver morpholinos at a throughput level of 25 consecutive injections per run of 2 min [98]. Recently an alternative robotic injection method was shown to inject embryos at a speed of 2000 per hour with a success rate of 99% [15]. This method makes use of specially designed grids where embryos occupy the hemi-spherical wells of an agarose cast in a centred and completely reproducible manner, with the cell mass always resting to the side. In this paper we present further applications and detailed methods for the use of this robotic injection system. We will also provide examples of high-throughput screening of injected embryos. Screening of zebrafish embryos can make use of the rapid technical advances in high-throughput analysis methods for zebrafish embryos [1,33,47]. The COPAS XL (Complex Object Parametric Analyzer and Sorter) system (Union Biometrica) can be used for fluorescence imaging of zebrafish embryos at a throughput level of 200 embryos per minute. This system has been designed for the analysis, sorting and dispensing of objects up to 1.5 mm in diameter based on size, optical density and fluorescence intensity. A profiler option simultaneously detects and analyzes up to 8000 data points per object for each of the channels of extinction and fluorescence, and includes advanced imaging options. The resulting profiles can be used to set parameters for zebrafish larvae to be sorted in 96-wells plates. In this paper we describe software to process the recorded data for further statistical analysis. Recently a vertebrate automated screening technology (VAST) with cellular-resolution and parallel animal processing has been reported in which the screening throughput is limited only by the image acquisition speed rather than by the fluidic or mechanical processes [16,67]. In the near future we will also use this methodology for zebrafish high content image analysis aimed at disease screening at high-throughput such as discussed below.

1.2. Applications of microinjection for studies of infectious disease

A growing list of pathogenic bacteria, filamentous fungi, yeasts, microsporidia, helminths, trypanosomes and viruses has been used for experimental infection studies in zebrafish, as detailed in several recent reviews [12,23,24,46,57,58,65,73]. Bacterial pathogens have been tested most frequently, as discussed by Meijer and Spaink [57] who present an overview of over 30 bacterial species for which disease studies in zebrafish have been published. In addition, zebrafish larvae have also been used to study the effects of a bacterial strains that normally do not cause a disease, *Staphylococcus epidermidis*, for the purpose of studying the effects of factors such as medical implant materials on defence mechanisms against commensal bacteria [97]. One of the most successful zebrafish disease models is the indirect study of human tuberculosis via the infection of zebrafish embryos with *Mycobacterium marinum*, as recently reviewed by Tobin, May and Wheeler [91]. The studies of *M. marinum* infection have already led to the clarification of many important processes in the life cycle of tuberculosis infection, in particular those underlying the mechanisms of granuloma formation in which the bacteria proliferate in macrophages [8,92]. The context of the embryo's developing immune system makes it possible to study the contribution of different immune cell types to disease progression already at 1–2 days post fertilization, when functional macrophages and neutrophil develop. Furthermore, due to the clear temporal separation of innate immunity from adaptive responses, zebrafish larvae are particularly useful for dissecting the innate host factors involved in pathology. Recent studies have underscored the remarkable similarity of the zebrafish and human immune systems, which is important for biomedical applications [94]. Since conserved pathogen associated molecular pattern recognition systems are already functional at one day post fertilization, zebrafish embryos and larvae are highly suitable for rapid screening of disease progression up to the stage that feeding becomes necessary and ethical constraints become apparent [85]. For infection studies a common route of infecting zebrafish embryos is the injection of the pathogen into the caudal vein of 1 day old embryos [7]. This method is relatively labour-intensive and although it has been successfully used for drug screens [86] it compares to cellular screening technologies as a low throughput technique, leading to major bottlenecks in drug discovery. Since infection by immersion in most infection models is not an effective alternative [96], we sought to achieve a reliable high-throughput automatic injection system, drastically reducing the man-hour requirement while vastly increasing the number of reproducibly infected embryos. As shown by Carvalho et al. [15] and Veneman et al. [97] we have successfully used robotic microinjection technology for screening bacterial proliferation during the first 5 days of larval development. In these studies the COPAS technology mentioned above was used to monitor bacterial proliferation. Carvalho et al. [15] have also shown that this high-throughput injection method, by its versatile applicability in small high safety flow cabinets, can be used for the study of infection by dangerous human pathogens such as *Mycobacterium tuberculosis* owing to the fact that this bacterial species can survive within macrophages of zebrafish larvae.

1.3. Applications of microinjection for studies of cancer progression

The zebrafish is increasingly used as a model for the study of cancer progression and metastatic potential of tumor cells [3,31,48,59,68,100]. The optical transparency of zebrafish larvae has permitted novel insights into mechanisms underlying tumor cell migration and the role of host recognition factors that are highly useful for the study of human cancers [32,32,39,90]. High-throughput methodologies have been applied in oncologic small molecules screens in zebrafish [80,88,89,93], however, testing has mainly been performed by addition of potential therapeutic drugs to the swimming water of zebrafish as microinjection of drugs has been hampered by technical limitations in the throughput level. However, the use of artificial carriers that can be used for slow release of drugs has been shown to be highly applicable for future screening for efficacy or toxicity of therapeutics in zebrafish [69]. Especially promising for disease studies in general is the use of photodegradable biogels that can be used for controlled release