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Embryonic zebrafish primary cell culture for transfection and live cellular and subcellular imaging

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ABSTRACT

Although having great potential for live cell imaging to address numerous cell biological questions with high spatial and temporal resolution, primary cell cultures of zebrafish embryos are not widely used. We present an easy-to-use protocol for preparing primary cell cultures of 2 dpf zebrafish embryos allowing for live cell imaging of fully differentiated cells such as neurons and myocytes. We demonstrate that different cell types can be identified by morphology and expression of transgenic cell type-specific fluorescent reporters and that fluorescent cells can be sorted by flow cytometry to prepare an enriched culture. To facilitate subcellular imaging in live primary cells, we successfully tested a selection of fluorescent vital dyes. Most importantly, we demonstrate that zebrafish primary cells can be transfected efficiently with expression constructs allowing for visualizing subcellular structures with fluorescent marker proteins for time lapse imaging. We propose zebrafish primary cell culture as a versatile tool to address cell biological questions in combination with a powerful *in vivo* model.

1. Introduction

The zebrafish (*Danio rerio, D. rerio*) is a model vertebrate becoming increasingly important for numerous fields of biomedical research (Ablain and Zon, 2013). The main advantage of zebrafish is the almost perfect transparency of early zebrafish embryos which develop rapidly outside the mother allowing for excellent *in vivo* time lapse imaging. In addition, manipulations of the zebrafish genome are relatively easy to perform (Sassen and Köster, 2015). In consequence, a broad collection of transgenic reporter lines exists with cell type-specific expression of various fluorescent proteins. Thus, cell populations and even whole organs can be observed in an intact developing organism and specific cell types can be identified and molecularly targeted by transgene expression. For example, fluorescent reporter lines have been successfully used to address diverse scientific questions such as the identification of different neuronal subclasses in the embryonic zebrafish brain to understand their development and function (Higashijima, 2008), microglial phagocytosis *in vivo* in intact brains of zebrafish larvae (Peri and Nüsslein-Volhard, 2008) or the identification of radial glia cells as progenitors of distinct neuronal and glial cell types in the zebrafish spinal cord (Johnson et al., 2016).

Although the early zebrafish embryo is almost transparent, visible light has a limited penetration depth and fluorescent emission is highly scattered when passing through tissue. This prevents efficient photon recovery from cells deep inside zebrafish larvae especially when small, sparsely labeled cellular structures are imaged. Moreover, due to a rapid embryogenesis, the organization of the different tissues is continuously changing thus making observation of cellular structures challenging. Even with highly advanced imaging systems, important details such as defined cellular morphologies, organelle dynamics or the subcellular localization of a zebrafish protein of interest in a specific cell type can sometimes be difficult to resolve. Further, the influence of defined biochemical factors on individual cell function, differentiation or behavior cannot be analyzed easily in whole mounts.

Some of these questions can be investigated either by using one of the few available zebrafish cell lines which are considered as heavily selected and modified (Driever and Rangini, 1993; Badakov and Jaźwińska, 2006; Senghaas and Köster, 2009) or by employing

Abbreviations: CMV, cytomegalovirus; dap, days after plating; DCX, Doublecortin; $DiOC_6$, 3,3′-Dihexyloxacarbocyanine iodide; dpf, days post fertilization; ER, endoplasmic reticulum; GFP, green fluorescent protein; *isl1*, *islet1*; PM, plasma membrane; *ptf1a*, *pancreas specific transcription factor 1a*; UAS, upstream activating sequence; VAMP1, vesicle-associated membrane protein 1

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immortalized mammalian cell lines (Manzoni et al., 2007). Yet, these two approaches may not reflect well the specific properties of a certain cell type of interest. Thus, it is preferable to use primary cells, which are cultured directly from the embryo and retain cell type-specific key features. Primary cell cultures derived from zebrafish embryos are easy and cost-efficient to prepare and have been used in the past to analyze the growth of neurons (Chen et al., 2013) or to screen for bioactive substances promoting cell differentiation (Huang et al., 2012; Xu et al., 2013; Ciarlo and Zon, 2016). However, zebrafish primary cell cultures are still far from widely used and their promising potential for live cell imaging on the cellular and subcellular level has still to be developed. One explanation for that could be the lack of reliable transfection protocols for primary zebrafish cells.

We present a simple and fast protocol for obtaining embryonic zebrafish primary cells. By exploiting the rich resource of cell type-specific fluorescent zebrafish reporter lines, we cultured and monitored different types of differentiated cells in detail, demonstrating that they maintain their original morphology in culture for several days, and we show that specific cell types can be enriched by flow cytometry prior to culturing. In order to facilitate subcellular imaging, we successfully tested several fluorescent vital dyes. Moreover, we have established a robust electroporation protocol allowing for single, double and triple plasmid transfections of differentiated primary cells for the visualization of organelles by the expression of fluorescent marker proteins. By providing an in-depth protocol, we would like to encourage the use of zebrafish primary cell culture as important research resource for cell biological questions complementing *in vivo* studies, thereby closing the gap between imaging of the whole zebrafish embryo and live cell imaging.

2. Results

2.1. Culturing primary cells from zebrafish embryos

An important prerequisite to successfully establish a primary culture is the sufficient sterilization of the donor tissue. To keep the number of germs and parasites low, zebrafish embryos were washed directly after spawning with Danieau 30% containing methylene blue, while subsequent raising was performed with daily medium exchanges. At 1 day post fertilization (dpf), the number of embryos was determined. At 2 dpf, embryos were enzymatically dechorionated, transferred into antibiotic-containing cell culture medium, deyolked and sterilized with ethanol. Next, embryos were dissociated into single cells by collagenase treatment and the cell suspension was filtered. Cells were plated on poly-L-lysine-coated glass bottom dishes (Fig. 1A) and incubated at 28 °C with daily medium exchanges. A detailed description of the complete procedure can be found in the Material & methods section of this manuscript.

The sterility of the culture was verified 1 day after plating (dap) by recording several transmitted light microscopy images of each dish. Thus, contamination with bacteria or other microorganisms could be easily detected. In these overviews, we could observe a large variety of cell morphologies with striated myocytes being most prominent (Fig. 1B,C,E). Staining with Calcein-AM, a cell-permeant dye which labels live cells with green fluorescence (Feller et al., 1995), showed that all adherent cells were viable. The strong fluorescence signal of Calcein-AM allowed for the observation of differentiated cells, *i.e.* polarized neuron-like cells, already at 1 dap (Fig. 1D). However, most primary cells do not have a long lifespan in culture (Orr et al., 1973)

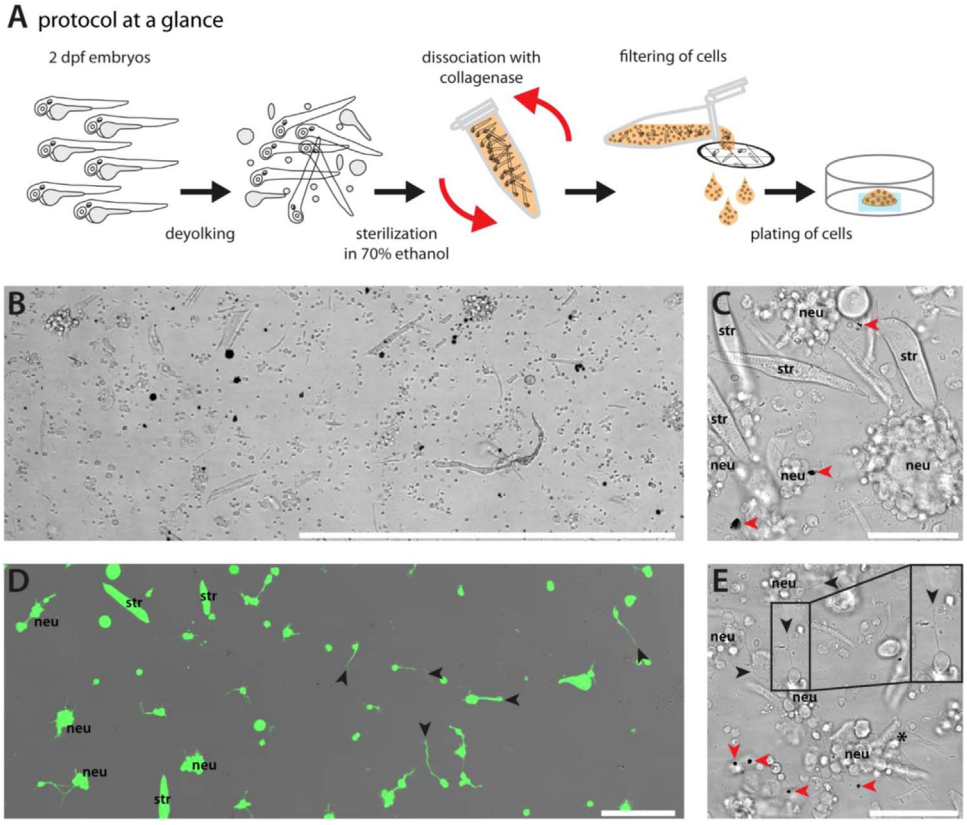

Fig. 1. Culturing embryonic zebrafish primary cells. **(A)** Depicted is a summary of the most important steps of the protocol. A detailed description can be found in the Material & Methods section. **(B)** Transmitted light image taken with a 10x objective showing primary cells in culture (1 dap) plated on a poly-L-lysine coated glass bottom dish. A mixture of different cell morphologies can be observed. Scale bar, 500 µm. **(C,E)** Transmitted light images recorded with a 63x objective of primary cells in culture (1 dap) displaying striated myocytes (str) and pigments (red arrowheads). Clusters of putative neurons (neu) spread fine processes (black arrowheads and inset in **(E)**). Also, a fibroblast-like cell (black asterisk) can be observed. Scale bars, 50 µm. **(D)** Merged image of transmitted light and green fluorescence channels recorded with a 10x objective of a typical culture stained with Calcein-AM to label viable cells at 1 dap. Scale bar, 100 µm.