Multiparametric analysis of neuromast hair cells in intact early larvae using a large particle sorter.

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Introduction

COPAS instruments automate the analysis, sorting, and dispensing of "large" objects such as viable small model organisms, seeds, beads, and particles, measuring the object size, optical density, and the intensity of fluorescent markers. Once analyzed, objects can be sorted according to user-selectable criteria, and then may be dispensed into stationary bulk receptacles or multi-well microtiter plates for high throughput screening. COPAS instruments have been proven to analyze and sort large objects with a higher speed and precision than present manual techniques. By automating the current, time consuming manual processes, the time required for experiments is dramatically reduced, human error is eliminated, and new experiments that previously could not be considered are now possible.

Fish have organs called neuromasts that detect the relative motion between the animal and their surroundings. The neuromasts have hair cells that can move. The hair cells are the mechanoreceptive component of the neuromasts. The movement of the hair cells triggers an electrical/neuronal signal to the brain where it is interpreted and processed. At four and five days postfertilization the zebrafish larvae have about 18 head and 9 trunk neuromasts along each side of the body. Lateral neuromast hair cells of zebrafish (*Danio rerio*) are extremely sensitive to aminoglycoside antibiotics such as neomycin. Neuromast staining patterns in larvae can be abolished by neomycin treatment. In this experiment, we tested the capability of the COPAS XL instrument in detecting neuromast-based staining patterns and the associated sorting ability in separating neomycin treated and untreated samples. We tested the capability of Profiler II, an add-on module for the COPAS instrument, in detecting the fluorescence signal along the longitudinal axis of the early larvae. This module measures positional fluorescent signals (peaks) along the length of the object. With Profiler II, it is possible to distinguish localized fluorescence from autofluorescence background, enabling the sorting of fluorescent objects containing even weak expression patterns.

Methods

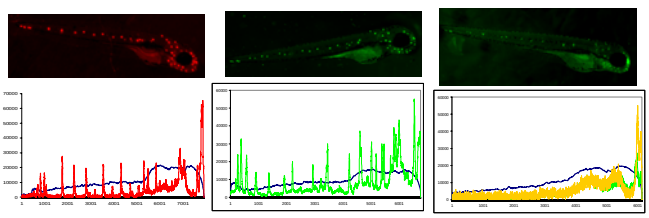
Materials and Methods

Wild type zebrafish larvae were purchased from Scientific Hatcheries (Huntington Beach, CA). Embryos were kept at 28.5ºC for three days after fertilization before an overnight delivery.
We thank Felipe Santos and Ed Rubel of the VM Bloedel Hearing Research Center at the University of Washington, Seattle, Washington for suggestions and protocols regarding the staining procedure. The following protocols are used for staining:
FM1-43 (Molecular Probes, T3163): 3 mM FM1-43 (stock made up in dH2O) in Embryo Media. Let the fish swim in the dye-embryo media mix for 30 seconds and rinse the fish 3 times in Embryo Media.
Yo-Pro-1 (Molecular probes, Y3603): 2 mM Yo-Pro-1 (stock is already made up in DMSO) in Embryo Media. Let the fish swim in the mix for 30min-1hr and rinse (3x) in Embryo Media
Daspei (Molecular Probes, D426): 0.005% Daspei mixed in Embryo Media for 15 minutes and rinse 3x.
For neomycin (Sigma Aldrich, N1142) treatment: 200 mM neomycin (the stock comes in dH2O) in Embryo Media. Let the fish swim for 1hour in the mix and rinse 3x.
Instrument: The COPAS XL was used for the experiment. Performance of the instrument was verified with control particles before the experiments.

Results

Detection of staining patterns

Zebrafish larvae (p4 or p5) were stained as described in the Materials and Methods. After staining, they were immediately run through a COPAS XL instrument. Three fluorescent dyes, FM 1-43, YO-PRO-1 and DASPEI were used for staining. As shown in Figure 1, lateral line neuromast specific staining patterns were observed for all three dyes. Fluorescence profiles generated by the instrument match with the staining patterns for both FM 1-43 and YO-PRO-1 (Figure 1A and 1B). However, fluorescence from DASPEI staining was too weak to be accurately detected by the instrument (Figure 1C).

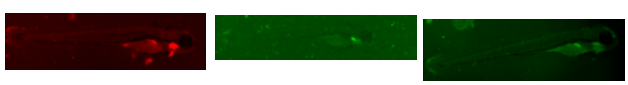


(A) FM 1-43 (B) YO-PRO-1 (C) DASPEI

Figure 1. Zebrafish larvae lateral line neuromast hair cell staining. (A) Stained with FM 1-43. (B) Stained with YO-PRO-1. (C) Stained with DASPEI. Blue line: Extinction (optical density) profile. Green line: green fluorescence profile. Yellow line: yellow fluorescence profile. X-axis: longitudinal axis of a zebrafish larva. Y-axis: fluorescence intensity or extinction.

Neomycin abolishes staining patterns

Treatment of the zebrafish larvae with neomycin abolished the staining patterns (Figure 2).



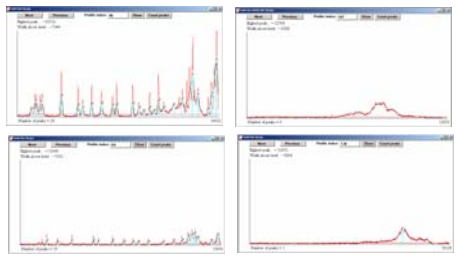
(A) FM 1-43 (B) YO-PRO-1 (C) DASPEI

Figure 2. Staining of neomycin treated zebrafish larvae.

Results

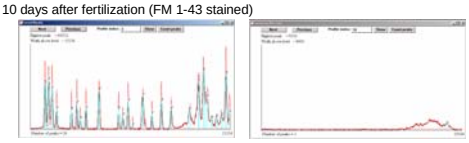
Peak number counting

5-day after fertilization (FM 1-43 stained)



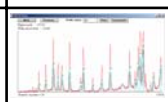
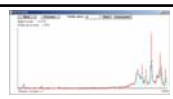
Fluorescent Nonfluorescent

Over 10 days after fertilization (FM 1-43 stained)



Fluorescent Nonfluorescent

Profiles with different peak counting numbers

	Peak number	Percentage	Profile
Strong peaks shown in the trunk	>15	54%	
Low peaks in the trunk	10-15	22%	
Peaks in the trunk not properly detected	5-10	24%	

Total: 78 (p5), FM 1-43 stained

The dispensing accuracy was checked by dispensing single larva into 96-well micro-titer plates and was verified to be almost 100% when examined under a microscope.

For 5 day sorting, 50 larvae were collected based on peak count numbers for either fluorescent or nonfluorescent population from a mixed sample (FM 1-43 stained fish with or without prior neomycin treatment). Examination of the collection verified that sorting was 100% accurate. Sorting criteria: fluorescent, peak number >5; nonfluorescent, peak number < 3.

For over 10 day sorting, a similar experiment was performed and 32 larvae were collected for either fluorescent or nonfluorescent population (FM 1-43 stained fish with or without prior neomycin treatment). Except for a dead fish with high autofluorescence among the nonfluorescent population, the sorting was 100% accurate. Sorting criteria: fluorescent, peak number >10; nonfluorescent, peak number < 5.

Conclusions

- COPAS XL instrument is able to detect neuromast cells based on staining patterns
- The instrument can analyze and sort neomycin treated and untreated samples.
- The Profiler II, an add-on module for the COPAS instrument, can detect the fluorescence signal along the longitudinal axis of the early larvae, up to 10 days postfertilization. This module measures positional fluorescent signals (peaks) along the length of the object.
- With the Profiler II, it is possible to distinguish localized fluorescence from autofluorescence background.

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