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Repeatability and Reproducibility of *In Vivo* Cone Density Measurements in the Adult Zebrafish Retina

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Abstract

Zebrafish (*Danio rerio*) are widely used as an experimental model for a wide range of retinal diseases. Previously, optical coherence tomography (OCT) was introduced for quantitative analysis of the zebrafish cone photoreceptor cell mosaic, however no data exists on the intersession reproducibility or intrasession repeatability of such measurements. We imaged 14 wild-type (WT) fish three times each, with 48 hours between each timepoint. *En face* images of the UV cone mosaic were generated from the OCT volume scans at each timepoint. These images were then aligned and the overlapping area cropped for analysis. Using a semi-automated cone-counting algorithm, a single observer identified each cone to calculate the cone density for every image, counting each image twice (84 total counts). The OCT cone density measurements were found to have an intersession reproducibility of 0.9988 (95% CI = 0.9978 – 0.9999) and a intrasession repeatability of 136.0 ± 10.5 cones/mm² (about 0.7%). Factors affecting image quality include gill movement during acquisition of the OCT volume and variable inclusion of non-UV cone mosaics in the contours used to generate the *en face* images.

XX.1 Introduction

Zebrafish (*Danio rerio*) are widely used to model human development and disease. Zebrafish have orthologs to many disease-causing proteins, whose functional domains are often similar to those found in human proteins (Langheinrich 2003; Howe et al. 2013). Zebrafish are especially valuable as an ocular model system since their cone rich retinas mimic the human retina (Allison et al. 2004) and multiple zebrafish models exist for ocular diseases (Link and Collery 2015). In addition, zebrafish are a cost-effective model for drug development, and have been used to accurately predict mammalian teratogenicity (Van Leeuwen et al. 1990) as well as drug oculotoxicity (Deeti et al. 2014), which is especially important given the number of drugs with ocular side effects (Santaella and Fraunfelder 2007). Finally, zebrafish mature quickly and breed prolifically, making them well-suited for high-throughput screening (Schutera et al. 2016; Truong et al. 2016).

Optical coherence tomography (OCT) is a non-invasive, high resolution imaging modality that has previously been used to examine zebrafish retina, lens, and anterior segment (Rao et al. 2006; Bailey et al. 2012; Collery et al. 2014). Recently, an OCT-based method for deriving *in vivo* estimates of cone density in the adult zebrafish retina was introduced (Huckenpahler et al. 2016). This method could allow researchers to quantify photoreceptor changes in response to disease or drug treatment, though the repeatability of these measurements has not been studied. Here we assess the intrasession repeatability and intersession reproducibility of OCT-based measurements of UV cone density in the adult zebrafish.

XX.2 Methods

Volumetric images (1000 A-scans/B-scan, 1000 B-scans, nominal scan width of 1.2×1.2 mm) of the zebrafish retina were obtained using a Biopogen Envisu R2200 SD-OCT (Research Triangle Park, NC) equipped with a 186.3 nm bandwidth SLD (center wavelength = 878.4 nm). Fourteen WT zebrafish were imaged three times each, with 48 hours between imaging sessions. *En face* images of the UV cone mosaic were generated from the OCT volumes as previously described (Huckenpahler et al. 2016). The *en face* images for each fish were manually aligned using Photoshop (Adobe Photosystems, San Jose, CA) and a common region of interest (ROI) was cropped (ROI size varied from 12.18–102×10⁻³ mm²). A low pass filter was applied to each ROI, and cones were identified using semi-automated cone counting software (Garrioch et al. 2012; Cooper et al. 2016a). Axial length values were obtained as previously described (Collery et al. 2014) and used to calculate the lateral scale of each *en face* image (Huckenpahler et al. 2016), thus enabling measurements of cone density. Cone density was taken as the number of cones with bound Voronoi cells divided by the total area of the Voronoi cells (Cooper et al. 2013).

To assess the intrasession repeatability, the cropped ROI from each imaging session was counted twice. The within subject standard deviation (S_w) was taken as the square root of the average variance across all fish (Bland and Altman 1996). The repeatability is calculated by multiplying S_w by 2.77 and the 95% confidence interval calculated with the formula:

$$CI_{95\%} = 1.96 \times \frac{S_w}{\sqrt{2n(m-1)}} \quad (1)$$

Where n is the number of zebrafish imaging sessions (42), and m is the number of counts for each image (2). The measurement error can also be estimated as $S_w \times 1.96$ (Bland and Altman 1996).

To calculate the intersession reproducibility of the cone density measurements, both cone density estimates at each time point were averaged and used to calculate the intraclass correlation coefficient (ICC) using R statistical package (The R Foundation for Statistical Computing, Vienna, Austria).

XX.3 Results

Qualitatively, we observed variability in image quality across fish and imaging sessions. Factors such as shadowing from the overlying blood vessels and movement of the fish during scan acquisition can result in distortions or missing data in the resultant *en face* images of the cone mosaic (**Figure XX.1**), though this rarely affected the ability to identify whether a cone was present or not. Across the 14 fish, the average cone density was found to be 20,588 cones/mm², with densities ranging from 12,326 to 39,254 cones/mm² (**Table XX. 1**). Differences between repeated measures of cone density ranged from 0–246 cones/mm² with an average within-fish standard deviation (S_w) of 49.1 cones/mm². Intrasection repeatability was 136.0±10.5 cones/mm² (about 0.7%). This means that the difference between two measurements for the same fish would be expected to be less than this value for 95% of pairs of measurements. The measurement error for this same data was determined to be 96.2 cones/mm² (about 0.5%), meaning that the difference between a given measurement of cone density and the true value would be expected to be less than this value for 95% of observations. Examining the cone density measurements over time, we found the ICC to be 0.9988 (95% CI = 0.9978 – 0.9999), indicating that 99.88% of the total variance is due to real differences in cone density across fish.

XX.4 Discussion

Our data demonstrate excellent repeatability and reproducibility of *in vivo* measurements of cone density, using *en face* images of the UV cone mosaic derived from volumetric OCT scans. However, this study has some limitations. First, the repeatability of the OCT density measurements will depend on the quality of the *en face* images. A low quality OCT scan with multiple breathing artifacts or low signal will produce a low quality *en face* image of the cone mosaic and subsequently impact the repeatability of the cone density measurements. Secondly, the repeatability and reproducibility measurements were performed by an expert who was proficient in both acquiring OCT images and generating *en face* images. For other observers, the repeatability and reliability of this technique may be worse (Bartlett and Frost 2008; Liu et al. 2014). Finally, our analysis was limited to the UV-cone submosaic- as other submosaics are more difficult to visualize (Huckenpahler et al. 2016); thus our results should not be assumed to apply to the R/G- or S-cone submosaic.

Adaptive optics scanning light ophthalmoscopy (AOSLO) is another technique capable of providing high-resolution *in vivo* images of the cone mosaic (Williams 2011), and has been used for examining cone density in a wide range of retinal diseases (Carroll et al. 2013; Roorda and Duncan 2015). Semi-automated techniques have shown an intrasection repeatability of 2.7% for measures of parafoveal cone density (Garrioch et al. 2012). Fully-automated methods have been shown to have excellent reproducibility, with one study reporting an ICC of 0.989 (Chui et al. 2013). Thus, the repeatability of our OCT-derived cone density measurements in the zebrafish retina are at least as good (if not slightly better) than AOSLO-derived measurements from the human retina. This may be due to the increased regularity of the zebrafish mosaic compared to humans, making it easier for the observer to see when the automated algorithm has missed a cone. In conclusion, with the non-invasive nature of the technique and availability of quantitative tools for analyzing

images, the OCT-based method described here could be used to quantitatively study ocular disease, monitor retinal development, and facilitate drug discovery in zebrafish with high reproducibility and repeatability.

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Fig. XX.1.

Disruptions in *en face* images of the cone mosaic derived from OCT volume scans. (A) Significant gill movements during the process of scanning result in missing data in the *en face* image (arrows). (B) Subtler gill movements and/or errors in contour placement can result in localized distortions (dashed rectangle). While these distortions may not affect the ability to identify the cones in the images, they would affect measurements of mosaic geometry (Cooper et al. 2016b). In addition, shadowing from overlying retinal vasculature was seen in many images. Scale bar = 100 μm .

Table XX.1

Zebrafish scaling information and density measurements

Fish	Axial Length (mm)	Region of Interest (μm^2)	Timepoint 1 (cones/ mm^2)		Timepoint 2 (cones/ mm^2)		Timepoint 3 (cones/ mm^2)	
1	1.41	49,644	20,527	20,482	20,632	20,683	19,496	19,401
2	1.60	12,185	19,980	19,986	20,614	20,734	20,684	20,893
3	1.91	42,025	12,453	12,479	12,578	12,593	12,563	12,526
4	1.88	29,649	18,589	18,557	19,047	19,170	18,711	18,664
5	1.39	50,392	26,185	26,231	25,810	25,756	26,400	26,439
6	1.56	98,882	15,238	15,224	15,450	15,427	15,248	15,281
7	1.60	38,325	12,711	12,645	12,435	12,373	12,761	12,802
8	1.56	64,947	18,939	18,926	18,669	18,687	18,792	18,756
9	1.75	102,133	12,339	12,394	12,472	12,447	12,368	12,326
10	1.69	75,105	13,038	13,031	13,477	13,495	13,260	13,260
11	1.66	50,265	15,067	15,089	15,071	15,068	14,875	14,817
12	1.26	24,073	28,636	28,513	28,113	28,095	28,016	27,993
13	1.11	20,022	35,651	35,405	35,508	35,531	35,828	35,836
14	1.22	35,429	38,598	38,587	39,229	39,254	38,717	38,666