Appendix S1

Background

Our samples were initially grouped into three different assays (referred to as Week 1, Week 2, and Week 3). Once imaging was complete, the samples were analyzed for batch-wise differences in brightness. Since upon initial analysis Week 3 had significantly lower brightness across all channels, that set of samples was re-run using backup slides (referred to as Week 3 RR). Appendix S1 compares Week 1, Week 2 and Week 3 RR, the three sets which were used for this study.

Capture Delta Statistics

Capture delta is here defined as the time in days between completion of the assay and imaging of a specimen in said assay. Delta values were obtained for all specimens with NBM images (N = 32). The NBM was selected because the histology was fairly consistent between specimens, and the images were guaranteed to include all channels. Positive controls could not be used because of inconsistent histology and sampling. The mean capture delta was 31.8 and the median capture delta was 30, with a range of [20, 52]. Plotted in a histogram, delta values appear to have a unimodal right-skewed distribution, which could interfere with the interpretation of some results here (See Figure 1).

One-way ANOVA was performed on Capture Delta by Assay Week, and a p-value of $<< 0.01$ was obtained. Tukey's HSD was used to obtain pair-wise p-values, and significant differences were found between *Week 1 & Week 2* ($p << 0.01$), and *Week 2 & Week 3* ($p < 0.01$). Weeks *1 & 3* were not found to differ significantly. See Figure 2 for a boxplot comparing the same variables.

Image Brightness Statistics

We used four measures of image brightness: 1) All channels, 2) DAPI only, 3) OXTR only, and 4) ChAT only. One-way ANOVA was performed on each measure of image brightness, followed by Tukey's HSD for pair-wise comparisons (See Table 1). A boxplot was also created to graphically compare image brightness across all four measures (See Figure 3). In both cases, no significant inter-group differences were observed.

Analysis of Capture Delta Effects on Image Brightness

We analyzed potential effects of differential capture deltas on specimen brightness across all four measures. Brightness values for all four measures were plotted and OLS trendlines were overlaid (See Figure 4). Although at first glance, there appears to be a consistent negative correlation between Capture Delta and brightness values, the histogram of Brightness across all channels (rescaled by minimum brightness in range) displays an apparent bimodal distribution (See Figure 5). This bimodal distribution is confirmed by a scatterplot of brightness values for all four measures overlaid with LOESS trendlines and colored by week (See Figure 6). The LOESS trendline and assay-wise coloring shows that the negative slope of the trendline is most likely due to outliers in Week 1 than to capture deltas. Univariate OLS regression was then performed for both Brightness by Capture Delta and Brightness by Assay Week across all four measures (See Table 2). In all cases, p-values for regression of Brightness on Capture Delta were insignificant with $p >> \alpha = 0.05$, and regression of Brightness on Assay Week also produced p-values $>> \alpha = 0.05$.

Summary of Results

This analysis showed no significant evidence of differential image brightness due to Capture Delta or Assay Week. This likely means that the issues seen in the initial Week 3 assay results were corrected by re-running the assay. That said, outliers from Week 1 may need to be carefully examined as part of the analysis, since Week 1 brightness appears to have greater variation than the other two weeks.

Tables

Brightness Measure	**Overall ANOVA p-value**	**Tukey's HSD p-value (Week 1-Week 2)**	**Tukey's HSD p-value (Week 1-Week 3)**	**Tukey's HSD p-value (Week 2-Week 3)**
All Channels	0.98	1.00	0.98	0.99
DAPI	0.394	1.00	0.46	0.51
OXTR	0.382	0.98	0.41	0.55
ChAT	0.90	0.89	0.98	0.95

Table 1: P-values for ANOVA and Tukey's HSD (Brightness Measure ~ Assay Week).

Brightness Measure	**p-value for *Brightness ~ Capture Delta***	**p-value for *Brightness ~ Assay Week***
All Channels	0.29	Week 2 = 0.95 / Week 3 = 0.84
DAPI	0.30	Week 2 = 0.96 / Week 3 = 0.23
OXTR	0.49	Week 2 = 0.85 / Week 3 = 0.21
ChAT	0.17	Week 2 = 0.66 / Week 3 = 0.87

Table 2: P-values for OLS regression models of brightness on either capture delta or assay week.

Figures

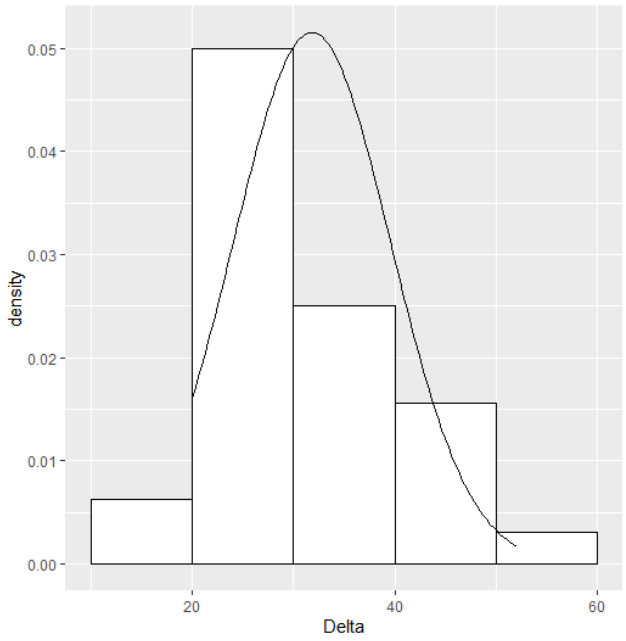

Figure 1: Histogram of Capture Delta values.

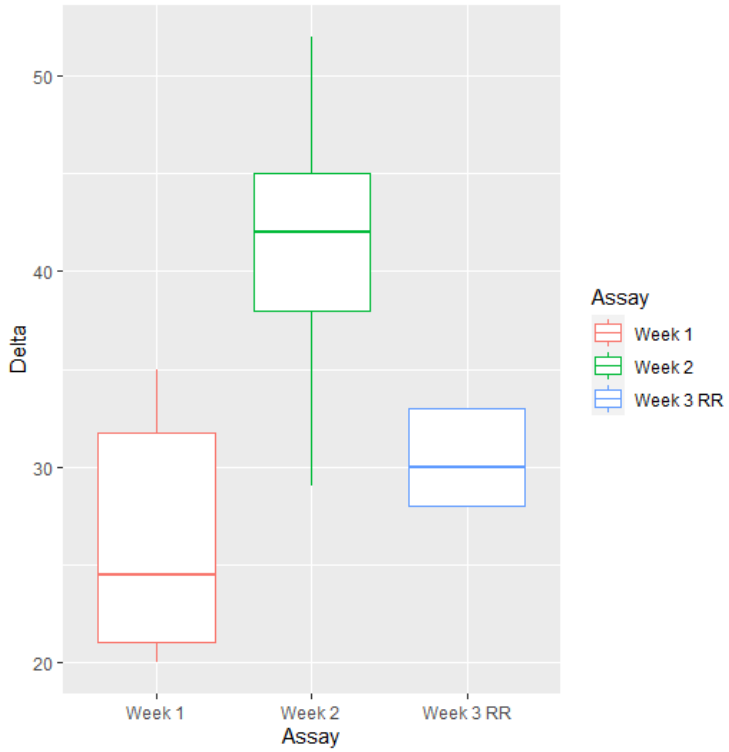

Figure 2: Boxplot of Capture Delta by Assay Week.

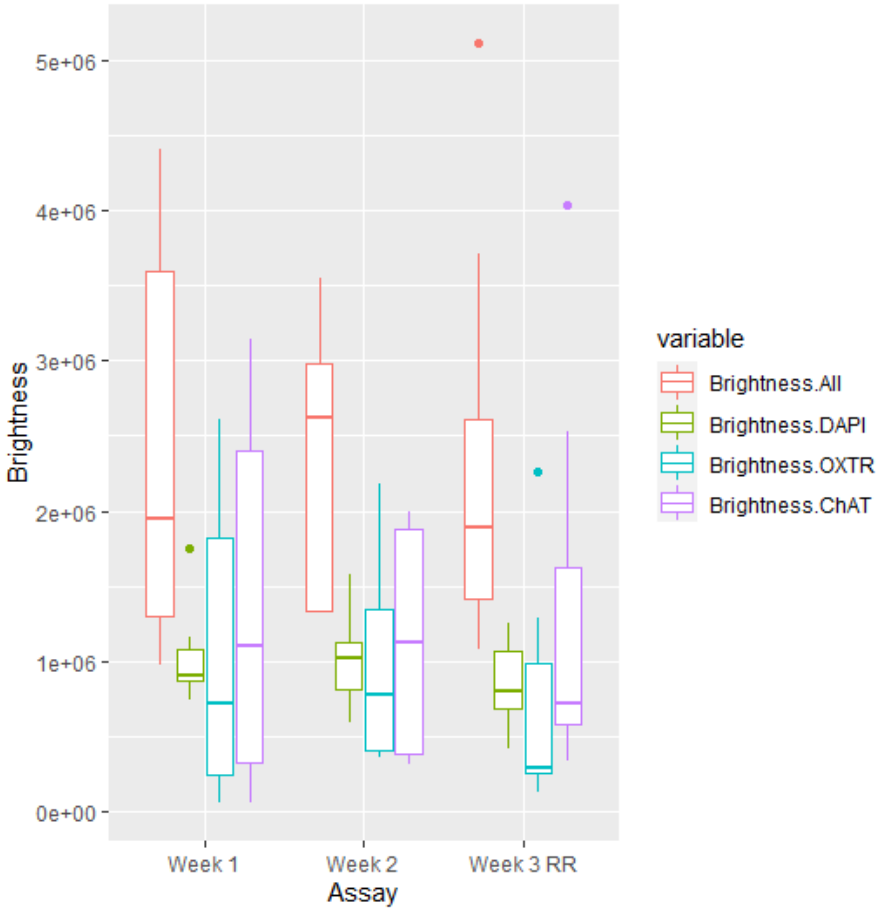

Figure 3: Boxplot of Image Brightness measures by Assay Week.

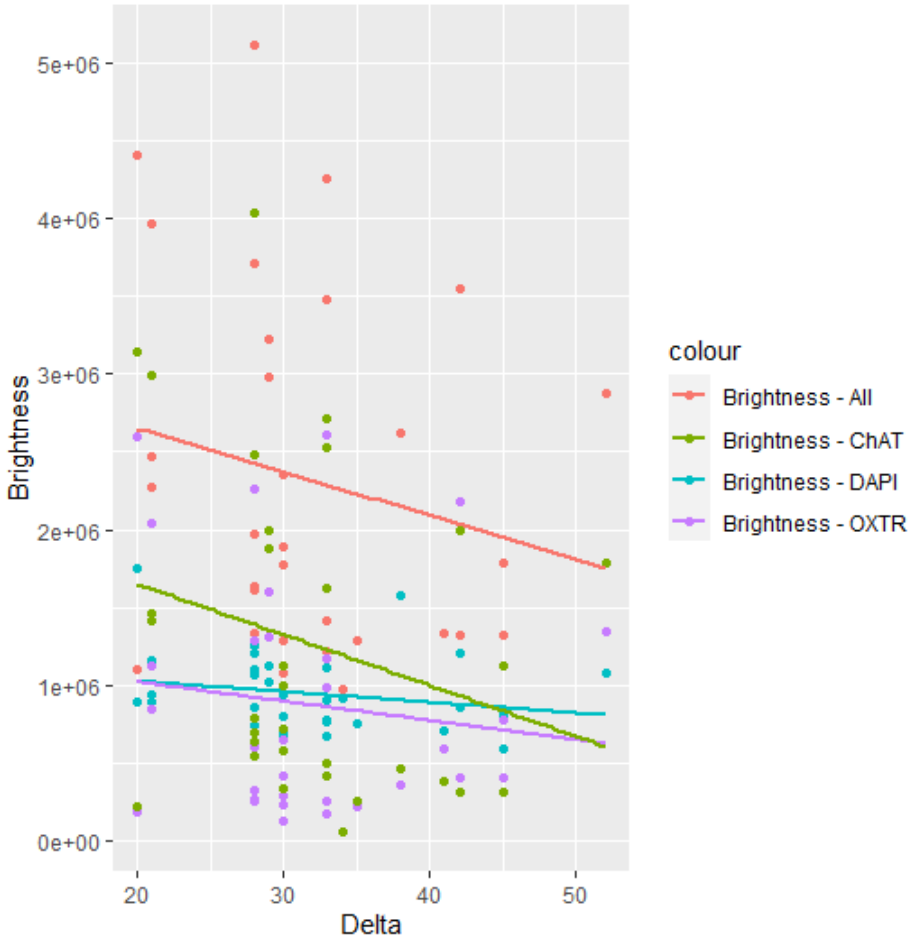

Figure 4: Scatterplot of Image Brightness with OLS trendline overlays.

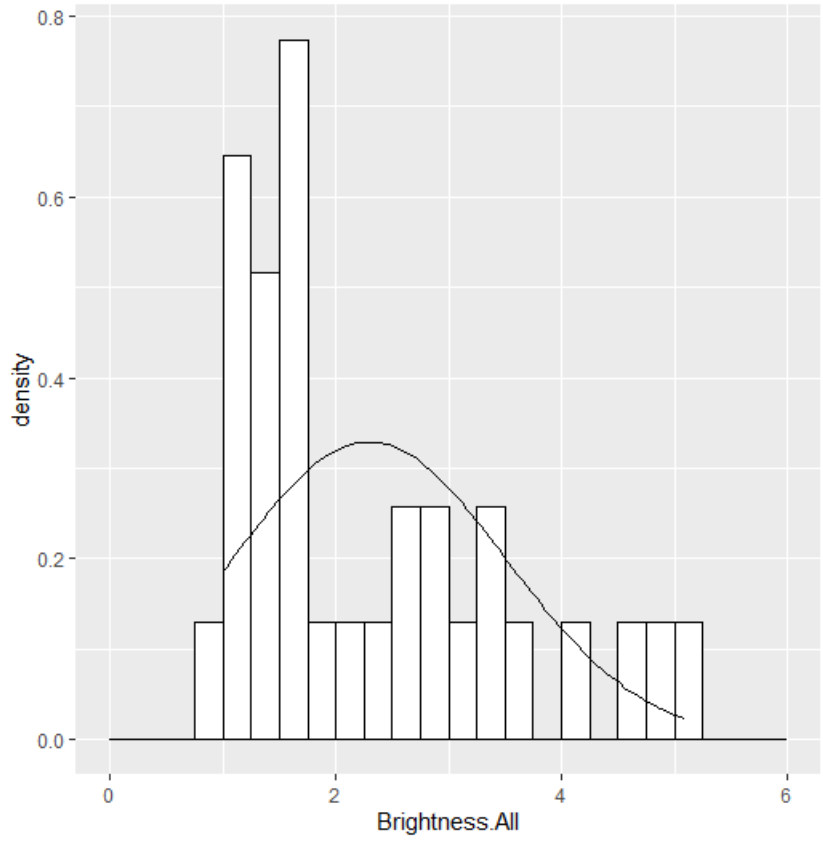

Figure 5: Histogram of Brightness across all channels values.

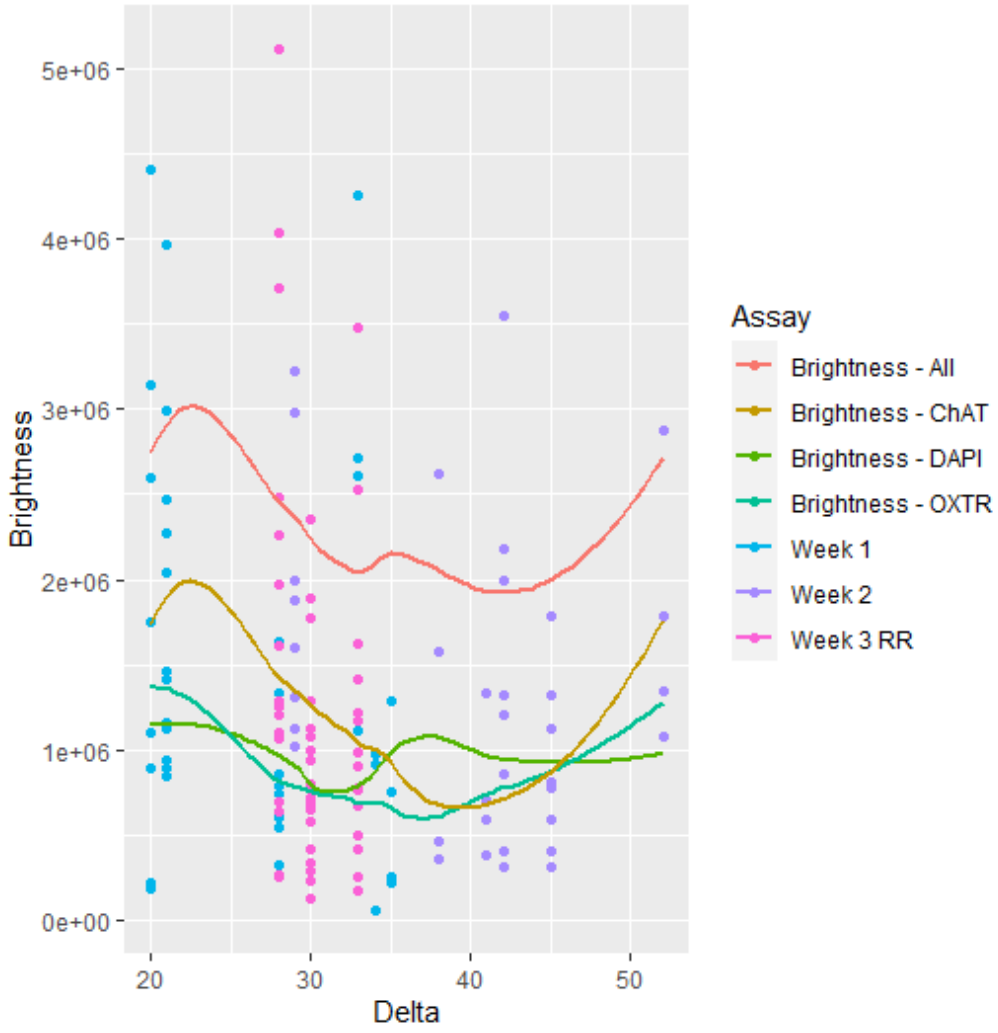

Figure 6: Scatterplot of Image Brightness measures, colored by Assay Week, with LOESS trendlines overlaid.