**AUTOFLUORESCENCE CHAPTER**

**Autofluorescence in C elegans**

<C elegans is great for quantitative experiments>

One major barrier in quantitative experiments using C elegans is autofluorescence. Whilst usually minor in red channels excited with \_ wavelengths, autofluorescence is particularly prominent in channels excited with blue wavelengths which are commonly used to image green fluorophores. When using endogenously tagged proteins, which are often expressed at low levels, this contribution is often be a significant fraction of the total signal, and can therefore significantly obscure the true signal that one is interested in. This might pose particular problems for quantitative experiments, where the absolute signal levels may be important.

We can observe this problem by imaging untagged control embryos. As shown in (fig x), a significant amount signal is collected in the GFP channel, which varies both spatially within the image, and between different images. By comparison, total signal in embryos endogenously tagged with LGL GFP is also highly variable, and only marginally higher than N2s, suggesting that a significant fraction of the total signal observed in these cells is autofluorescence, and that the intra-embryo signal variation is largely due to variable autofluorescence. Despite being enriched on the posterior cortex, which is easily visible in cells with overexpressed LGL (ref), this is difficult to visualise here as a result of autofluorescence. Therefore, if we want to accurately visualise, and indeed quantify, protein levels and distributions, we need a method that can locally correct AF on a pixel-by-pixel basis.

One approach that has been used for this is spectral imaging. Typically used to separate overlapping fluorophore signals based on spectral characteristics, this approach can also be used to separate out autofluorescence by treating it much like a fluorophore with its own spectral characteristics. Whilst often effective, these techniques require specialised instruments and analysis tools and cannot be performed on standard confocal microscopes.

However, simpler approaches have been used. By exploiting the fact that autofluorescence can often be described as a single component, with an emission spectrum much broader than GFP, one can find an emission wavelength (usually red) that is specific for autofluorescence, and use this channel to infer the amount of autofluorescence in the sample. This can then be subtracted away from the fluorophore channel, giving a ‘clean’ readout of fluorophore signal. In comparison to full spectral imaging, this method can be carried out with standard light sources and emission filters, and therefore can be easily implemented into existing workflows.

Inspired by this work, we aimed to implement, and assess the applicability of such a method to images of C elegans zygotes. In doing so, we have put together a robust and easily-implementable workflow which we’ve termed SAIBR: Spectral Autofluorescence Image correction by regression.

**SAIBR: a simplified method for autofluorescence correction based on dual emission imaging**

At minimum, autofluorescence correction relies on the ability to find an ‘autofluorescence-reporter’ channel that is free of GFP signal, but rich in autofluorescence, such that this channel can be used an independent readout of autofluorescence in the sample. Full spectral analysis performed by Nelio Rodrigues (not shown here), shows that red shifted emission filters, which are commonly used to image red fluorescent proteins, meet such a requirement.

Furthermore, by imaging untagged embryos with both the standard GFP channel and the AF channel, we find a strong linear correlation between pixel data from the two channels. Whilst raw pixel values do not correlate well, as these are dominated by noise, we can get a strong correlation by first applying a Gaussian filter to suppress this noise (fig x). We found that this relationship is consistent between embryos (fig x b, c). Furthermore, we found a near identical relationship when plotting the mean intensity values of individual embryos, suggesting that the same relationship can account for both intra- and inter-embryo AF variation.

Together, this implies that taking an autofluorescence channel image is sufficient to accurately predict the level of autofluorescence in the GFP channel. To quantify the necessary inter-channel conversion factor, I performed linear regression, using an ordinary least squares method, on Gaussian-filtered pixel values pooled from multiple untagged embryos.

Then, to perform correction on images containing fluorophore, we just need to capture an autofluorescence channel image, alongside the GFP channel image, rescale the image according to this predefined relationship, and then subtract this away from the GFP channel image.

**Autofluorescence correction using SAIBR**

To assess the effectiveness of SAIBR, and it’s utility in the analysis of PAR proteins, I applied it to a range of images of unlabelled and GFP-labelled embryos. As expected, applying SAIBR to images of unlabelled cells reduced fluorescence from across the cell to zero, with no visible structures remaining. This is a good validation of the method, and suggests that it can properly account for all of the autofluorescence in the cell.

As already shown, images of LGL are dominated by autofluorescence, and so SAIBR was expected to be particularly useful. As shown in fig x, SAIBR strongly reduces signal within the cell, and improves contrast at the posterior cortex, allowing us to better resolve cortical enrichment at the posterior. Improvements are similar for PAR-3. In addition to improvements at the cortex, we see that SAIBR can suppress the local fluorescence minimum at the cell centre caused by lower AF at the pronuclei. For PAR-6 the improvements are qualitatively less striking, due to a higher ratio of fluorophore signal to autofluorescence, but nonetheless quantitatively important.

As shown in fig x, SAIBR has a strong impact on the shape of intensity profiles taken across the cortex within each polarity domain, in all cases showing a clearer peak and suppression of signal at the internal portion of the curves. As discussed in the next section, this has a particular importance for quantification of membrane and cytoplasmic concentrations.

**Extending SAIBR to dual-labelled C elegans embryos**

As SAIBR relies on a red shifted emission channel, complications can arise when there is a red fluorophore present. As red fluorophores are usually weakly excited by blue lasers, they will contribute additional signal to the AF channel, which may lead to overestimation, and therefore oversubtraction, of autofluorescence if not accounted for. If RFP levels are low, this effect may be small and can be ignored. However, if RFP levels are high, this bleedthrough effect can be significant. This can be demonstrated by observing the inter-channel relationship in control embryos tagged with a red fluorophore (fig x). We find that, when an RFP is present, this relationship deviates significantly from the typical relationship observed in N2s, in direct proportion to local RFP levels (fig x inset). As this relationship is linear, autofluorescence in the GFP channel can now be described as a linear function of both the AF and the RFP channels. Plotting the pixel data in three dimensions shows that the data can be successfully fit to a plane, by performing multiple linear regression (fig x).

Then, to perform correction on images containing fluorophore, we just need to capture all three channels, calculate autofluorescence using the three-channel regression relationship obtained from the appropriate RFP tagged single line, and then subtract this away from the GFP channel image. This is demonstrated in figure x, for embryos expressing both PAR-6 GFP and MEX5 mCherry, or just MEX5 cherry. Whereas 2-channel SAIBR results in oversubtraction of autofluorescence (particularly visible in the MEX5 cherry single line), this is eliminated when using 3-channel SAIBR.

**Discussion**

In summary, I have demonstrated that a simple protocol, which we’ve termed SAIBR, can be used to successfully correct autofluorescence in images of C elegans zygotes. The improvements are particularly striking for images of fusion proteins with low levels of expression, such as LGL, but even when expression levels are higher, such as PAR-2, AF correction will prove important for quantitative analysis, as discussed in the next section.

The simplicity of the method means that it can be easily incorporated into existing workflows, and should be applicable to a variety of imaging platforms. In the full study, we showed that the method is equally successful on both spinning-disk confocal and wide field instruments.

Whilst designed with C elegans in mind, the method is <>, and could be applied to a number of other model systems in which autofluorescence is a problem. In the full study, we have shown that the method works successfully in later developmental stages in C elegans, as well as other model organisms such as starfish and yeast. That said, the method isn’t guaranteed to perform well in all cases. If samples contain multiple, independently varying sources of autoflourescence, then SAIBR may face problems as a single autofluorescence reporter channel cannot account for this. However, much like how we can tackle red fluorophores, we have found that in some cases this can be solved simply by adding one extra reporter channel. Inevitably, though, such an approach may not be compatible with dual-colour imaging.

Whilst the analysis steps are relatively straightforward, implementing the computational workflow may still be a barrier to adoption for some users. Therefore, to make the protocol accessible, I have put together a simple GUI-based FIJI plugin which can carry out all the analysis steps in a few simple steps. This can be found here:

<https://github.com/tsmbland/saibr_fiji_plugin>.

The method comes with a few tradeoffs, which will vary in significance depending on the particular study. One issue is that, as the method combines pixel noise from multiple images, corrected image can in some cases be quite noisy, particularly where weak imaging conditions are used. It also requires capturing two emission channels for each image, which doubles sample illumination times and potential phototoxicity, which may be an issue for long timelapses. Additionally, if samples display rapid motion, then the time lag between taking these two channels may lead to pixel mismatches, which could introduce artifacts. These last points could be fixed by using am imaging setup that allows for dual capture of multiple emission bands. For this particular study, none of these issues will be of major significance.