**Methods for quantification of membrane concentrations in C elegans zygotes**

<intro>

A number of methods have been implemented aiming to quantify cortical protein amounts in C elegans embryos. Some methods quantify membrane concentrations by finding the region of the image representing the cortex (either by manual or computational segmentation) and extracting pixel concentrations within this region (fig \_). Goehring manually segmented the embryo cortex, computationally straightened a region around the cortex, and summed the highest intensity group of pixels within this region. Hubatsch used a similar approach, but replaced manual segmentation with an automated computational pipeline. Similarly, Zhang used an elaborate protocol to computationally segment images, and defined cortical concentrations as the average signal intensity within a region representing the cortex.

A main disadvantage of these methods is that they fail to account for the fact that much of this signal will always be derived from cytoplasmic protein (and indeed autofluorescence as none of these methods (?) have used spatial autofluorescence correction). This means that measures of membrane concentration are sensitive the changes in cytoplasmic concentrations, and means that the methods fail to achieve an accurate zero (positive signal will still register in cases where there is nothing on the cortex). Typically, attempts are made to overcome this latter point by normalising concentrations and/or subtracting away a local or global estimate of the background signal, but this is often difficult and inaccurate.

More advanced methods have aimed to overcome this problem by using an optimisation procedure to fit the shape of the cross-cortex profile to a model composed of cytoplasmic and membrane contributions. This requires prior description of the expected form of cytoplasmic and membrane signal contributions. Such an approach was used by Gross, who described the cross-cortex profile at each point around the circumference of the embryo as the sum of a gaussian and an error function contribution, representing the expected form of a point (cortex) or step-function (cytoplasm) convolved with a Gaussian-like point spread function in 1D. Cytoplasmic and membrane concentrations can then be found by fitting measured profiles to this model and extracting the relevant parameters describing the amplitudes of the two signal components (fig \_). The model also includes a parameter describing the position of the cortex, which can be optimised to align the model to the profile, eliminating the need for accurate prior segmentation. A similar approach was previously used in Blanchoud, but they used a <> instead of a sigmoidal cytoplasmic contribution.

The assumption that cytoplasmic and membrane contributions can be described by such simple mathematical functions may be far from the truth. This was demonstrated by Reich. Rather than assuming what a cross-cortex distribution of cytoplasmic protein would look like, Reich used images in which all tagged protein is cytoplasmic to directly measure the contribution of cytoplasmic protein to a cross-cortex profile, finding that (under imaging conditions similar to those used in this study) the shape deviates significantly from an error-function. Specifically, whilst similar to an error-function, cytoplasmic signal continues increasing significantly far into the embryo (\*which can be seen in figure A.3 of Jake’s thesis, and later replicated in figure \_ of this study, although it’s worth noting that the original study didn’t use spatial autofluorescence correction, so there may be some artefacts relating to this).

<Good place to put cytbg figure>

The main reason for this is likely due to scattering and diffraction of light from planes above and below the imaging plane, combined with a curved geometry in the z-dimension (fig x). Scatter, which is a common issue in images of biological samples, is a broadening of light in three dimensions as it passes through regions of heterogeneous refractive index. This occurs within the (xy) plane of an image, but is typically far more significant in the z-plane, meaning that pixel intensities within a plane will be affected not only by structures within that plane but also structures above and below.

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Typically, one can account for this by taking a z-stack from across the sample, and applying a deconvolution algorithm to the 3D stack to reassign all blurred/scattered light to an in-focus location. These methods rely on prior knowledge of the point spread function that applies to the particular sample and imaging set-up. This needs to be as accurate as possible, otherwise artifacts can result.

Theoretical methods exist to estimate an appropriate PSF given parameters such as the imaging modality, NA and emitted light wavelength. Whilst this can accurately describe blur within the imaging apparatus, scattering within the sample and at the sample-apparatus interface is difficult to model. For this reason, it can be more effective to measure an empirical PSF by imaging the light distribution from a single point source (e.g. a fluorescent bead) under similar sample prep conditions to your sample. The accuracy of this depends on how closely the environment can be replicated, which is difficult. <soaking tissue with beads>. Furthermore, most deconvolution methods assume that the PSF is a constant function throughout the whole image, but in many cases this won’t be the case. Such may be the case if there are refractive index gradients within the sample. Additionally, if there is a mismatch between the refractive index of the immersion and mounting media, as is often the case, then the PSF will vary with depth as spherical aberrations will be introduced deeper into the sample. A fluorescent bead located directly below the coverslip will not capture this.

In reality, given all of these confounding factors, an accurate description of the PSF that applies to a given sample of interest is often an unachievable goal. For this reason, I opted against using a deconvolution approach to account for out-of-plane scattering.

Fortunately, in this particular case, matters are greatly simplified by the fact that the geometry of embryos are highly consistent. Not only is the shape of embryos highly consistent, but PAR protein distributions also tend to display rotational symmetry (at least during normal polarity development in P0) meaning that protein distributions in planes above and below the focal plane tend to be similar/identical to those seen at the focal plane (much like the simulations in figs x and x). Optical properties are also not expected to change from sample to sample, or from location to location around the circumference of an embryo. Together, these features imply that, for cytoplasmic or membrane protein, the normalised shape of the cross-cortex profile measured at the midplane should be some consistent function, that shouldn’t vary much between embryos or spatially around the circumference of an embryo. A change in local or global concentration should amount to a rescaling of this profile, but shouldn't change the normalised shape. Where protein is both cytoplasmic and cortical, the total measured cross-cortex profile will then be a sum of these two contributions.

Thus, a key step in the path to accurate quantification is the ability to measure these reference profiles. As mentioned previously, cytoplasmic reference profiles can easily be obtained by analysing cells in which all protein is cytoplasmic. Indeed, Reich used an approach half-way between the Gross method and the method that I am proposing, replacing the error-function description of cytoplasmic signal with his arbitrary, measured, cytoplasmic reference profile. Thus, total signals were fit as the sum of a Gaussian profile and this arbitrary cytoplasmic reference profile, which resulted in a far better ability of the model to fit the shape of measured profiles.

Whilst this move is a significant step in the right direction, the problem remains of how best to account for out of focus cortical light. This presents a challenge: whilst it is relatively easy to directly measure a cytoplasmic reference profile (you just need a reference image in which all signal is cytoplasmic), the same is not true for a membrane profile as it is difficult/impossible to find a reference case in which all protein is membrane bound. To extend the Reich method to account for out-of-focus membrane protein, I attempted to find alternative methods to get an approximation of the membrane reference profile.

In fact, this problem becomes simplified when one considers that, even in cases where the cortex is polarised, the cytoplasm of most PAR proteins should be uniform (although exceptions do exist in the case of PAR-1 and PAR-3, where true cytoplasmic gradients have been observed, although the basis for this is poorly understood). Thus, straightened cortices can be modelled as a uniform cytoplasmic component, defined by a cytoplasmic reference profile (Rc) and a single, uniform, cytoplasmic concentration (C), plus a polarised membrane component, defined by a membrane reference profile (Rm) and a nonuniform membrane concentration profile (M). This is shown in fig x.

Since Rc is predefined and, if we assume for a second that Rm is also predefined, then M and C for a given embryo can be determined by fitting a straightened image to this model. (At this point these values will be in arbitrary units, but I’ll come back to this point). Whilst Rm is in fact not predefined, given the constraints imposed by cytoplasmic uniformity, it arises that only a model with an appropriate membrane reference profile will be able to create simulated images that closely match experimental images. (Imagine, for example a model with a Gaussian membrane reference profile, which would clearly fail to capture the graded internal signal). Therefore, under conditions such as these, in which we have a uniform cytoplasmic component and a graded membrane component, the membrane reference profile need not be predefined, and, in theory, can be fit to the data along with the concentration parameters. To perform this kind of optimisation, I have used a gradient descent approach based on differentiable programming, which is described below.

**A gradient descent protocol for image quantification**

Gradient descent is a popular optimisation strategy used for a number of machine learning applications. The idea is to calculate the partial derivative of each input parameter with respect to a loss term (mean squared error). A negative gradient for a parameter would imply that an increase in that parameter would decrease overall loss, whereas a positive gradient would imply the opposite. Therefore, to reduce loss, each input parameter can be adjusted according to its partial derivative. Starting with a set of initial conditions, this procedure is then iteratively repeated, adjusting parameters and calculating new gradients at each step, until the loss term reaches a minimum.

The utility of these methods has been greatly advanced in recent years by the advent of differentiable programming tools. Commonly used for deep learning, although generalisable to other problems, these tools greatly speed up computation for complex optimisation procedures by automatically calculating gradients at every step, rather than relying on numerical methods, using a process called backpropagation. In addition, extensions to the basic gradient descent algorithm (e.g. Adam) have proven effective at speeding up convergence and preventing entrapment in local minima.

In the case of this particular problem, this procedure is described in figure x. Given a set of parameters (M, C, Rm, Rc), a forward propagation step simulates an image (fig x), and then this is compared to a ground truth image to calculate an error term. Backpropagation then calculates the gradient of each of the input parameters with respect to this error term. At this point, we can adjust some or all of the input parameters according to these gradients. Repeating this cycle of forward and back propagation will then lead to a gradual optimisation in these parameters, until a minimum is reached.

To test this approach, I first applied it to images of polarised PAR-2. Built using the differentiable programming package Tensorflow, the model was initiated with all concentrations (M and C) equal to zero, and Rm initiated as a Gaussian. For Rc I used a measured profile (fig x), and this was not adjusted during training (fig xa). Using an Adam optimiser with a learning rate of 0.01, all other parameters (M, C and Rm) were then adjusted iteratively until a plateau was reached (250 steps), as shown in fig xb. As shown in fig xc, the final simulated image, composed of a uniform cytoplasmic component and a nonuniform membrane component, fits closely to the ground truth image.

**Segmentation**

In addition to the parameters already mentioned, the model also includes a series of alignment parameters, which are also trained by gradient descent, allowing the model to freely align to the data in the x direction. As a result, ground truth images do not need to be accurately segmented prior to optimisation, and rough manual ROIs are fine to use. Another nice outcome of this is that these offset parameters can then be used to refine the original ROI, meaning that the method can serve as a tool for computational segmentation. Refined ROIs can then be used to restraighten the cortex, and optimisation repeated.

**Benchmarking the method**

The method described so far is limited to a special case of images with polarised membrane concentrations. However, as discussed previously, the optimised membrane reference profile, which is a function of local geometry and optical properties, should be applicable to all embryos. Therefore, much like we used a predefined cytoplasmic reference profile when fitting polarised PAR-2, images of proteins without a polarised membrane can be quantified by using an Rm derived from a calibration procedure on polarised images.

To test this method, I performed quantification on images of PH with variable expression levels, obtained by performing an RNAi rundown using XFP (see Methods). In this case, I used a predefined Rm and Rc, and only optimised M and C (as well as the alignment parameters described in the previous section), fig xa. Compatible with expected linear membrane binding kinetics, we can see that the method gives a tight linear relationship between cytoplasmic and membrane concentrations. N2s are also accurately describes as having cytoplasmic and membrane concentrations of zero.

I next investigated how robust the method is to changing signal-to-noise ratios, using three PH embryos with varying levels of expression. Adding pixel noise to these images may be expected to add noise to the resulting concentrations, but shouldn’t bias the data in any direction. As seen in figues xc and xd, this is precisely the case for all three PH embryos. Quantification of N2s is also not biased by noise (fig xc,d grey points). In addition, segmentation results are strikingly robust to noise, even at high noise levels that render embryos near-invisible to the human eye (fig xb).

**Calibrating concentration units**

As M and C are in arbitrary units (effectively in units of their own respective reference profile), a conversion parameter, is required to put them in comparable units. To calibrate this conversion parameter, I quantified the effects on M and C measurements of redistributing a fixed pool of protein from the cytoplasm to the cortex. To do this, I used an optogenetics system with a membrane bound PH::eGFP::LOV to move a cytoplasmic pool of ePDZ::mCherry to the membrane (Fielmich et al., 2018). Embryos were exposed to blue light for 10 seconds, which promotes binding between ePDZ and LOV, leading to a rapid uniform recruitment of ePDZ::mCherry to the membrane and a reduction in the concentration in the cytoplasm (fig x). Where ePDZ mcherry is expressed alone, this localisation shift isn’t observed (fig x, grey).

Whilst there is significant relocalisation, the total amount of protein can be assumed to be constant. This total amount can be expressed as the C value that would be expected if all tagged molecules were in the cytoplasm, given by equation x where $\psi$ is the surface-area to volume ratio of the cell. Given that M is in different arbitrary units, a conversion parameter, c, is required:

X can then be calculated from the optogenetics embryos, on an embryo by embryo basis, by comparing the gain in M to the loss in C:

eq

Performing this analysis gives a value of x = x+- x, which can be used to put M and C in common units. Note that these concentrations are still arbitrary in the sense that they give no indication of absolute concentrations (i.e. absolute number of molecules per unit area). For much of the analysis in the following sections, where the aim is to measure membrane affinities (membrane to cytoplasmic ratios) this will not be an issue, although I’ll return to this point in section x.

**Discussion**

Accurate quantification of features from images relies on the ability to separate overlapping signals and correctly attribute signals to their source. In this section, I have described a two-step pipeline designed for accurate quantification of cytoplasmic and membrane concentrations from midplane images of C elegans zygotes. The first step involves separation of autofluorescence and fluorophore signal, and the second step involves separation of signals from cytoplasmic and membrane protein. The overall pipeline is not specific for any particular microscope, and makes no assumptions about the spectral characteristics of the signal components or the optical properties of the imaging system/sample. Whilst the SAIBR method isn’t fundamentally linked to C elegans, and has been shown to apply to other systems, separation of cytoplasmic and membrane signals is less generalisable, and a number of assumptions in the model presented here are firmly linked to the simple and reproducible geometries of C elegans zygotes and PAR protein patterns. Nevertheless, the ability to confidently quantify relative membrane and cytoplasmic concentrations in vivo brings forward new experimental possibilities for studies of the C elegans PAR network, and will prove fundamental to much of the work presented in later chapters of this thesis.