**Advantages of spinning disk**

Confocal microscopy prevents out-of-focus light from being detected by planning a pinhole between the objective lends and the camera.

Trade-off between phototoxicity, blur and noise

**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.

<physics/optics>

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.

<Transition>

As the normalised shape of this profile is a function only of local embryo geometry and the optical properties of the microscope/sample, both of which should be largely consistent from point to point around the circumference, and from embryo to embryo, an explicit description of light scattering is not necessary, and it should be sufficient to rely on a phenomenological description of the cytoplasmic signal contribution. Reich exploited this idea by replacing the error-function description of cytoplasmic signal with his arbitrary, measured, cytoplasmic reference profile, thus describing the total signal as the sum of a Gaussian profile and this arbitrary profile. The result was 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, an analogous problem remains in the case of the membrane signal contribution. After all, if the cytoplasmic contribution is significantly affected by out of focus light, then it’s likely that a similar problem applies in the case of membrane signal. <figure \_>. Specifically, it is expected that out of focus cortical signal might lead to a shape resembling an asymmetric Gaussian, with higher signal on the inside than the outside. In fact, this phenomenon can be easily observed just by looking at images of polarised PAR proteins (<reference earlier image>), where out-of-focus cortical signal can create the illusion of a strong cytoplasmic gradient (by comparison, two-photon images, which aim to eliminate out of focus light, show a completely flat cytoplasm (Petrasek), which is expected for most PAR proteins based on fast measured diffusion rates). Whilst in some cases this may be of little concern, problems can arise if accurate cytoplasmic quantification is required. Without accurate cytoplasmic concentration, measures of membrane to cytoplasmic ratio, which is often used as a proxy for membrane affinity (see later section), may be wildly off. This will prove significant for this study.

In this study, I aimed extend the Reich method by additionally striving for a more accurate description of the membrane signal contribution. This presents a unique 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 where all the protein is membrane bound.

<Eggshell method> An initial idea, involving staining of the exterior of the eggshell with a fluorescent dye proved technically challenging and not reproducible.

To get to this point, let’s first assume that the shape of the membrane and cytoplasmic reference profiles are known (or perhaps just assumed in the case of Gross)

**Calibrating model by gradient descent**

**Quantification by differentiable programming**

In the past, methods have typically considered each cross-cortex profile in isolation, performing independent optimisation processes on each. An alternative solution is to consider the entire straightened image (or a subset of profiles) together, aiming to optimise the fit between a model of the straightened cortex and the actual image.

**Segmentation**

A nice outcome of this is that the offset parameters can then be used to refine the original ROI. As a result, initial ROIs need only be very rough (4 haphazard clicks around the cortex generally suffices, compared to ~20 which may be required to accurately trace the cortex). Whilst this may seem like a minor point, the time saved from by this feature, in a project in which <several hundred> images have been quantified, has been immense (not to mention the reduced risk of repetitive strain injury)

**Benchmarking the method**

PH rundown

Noise