Quantifying spatiotemporal gradient formation in early *Caenorhabditis elegans* embryos with lightsheet microscopy

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Abstract

Major steps in embryonic development, e.g. body axes formation or asymmetric cell divisions, rely on symmetry-breaking events and gradient formation. Using three-dimensional time-resolved lightsheet microscopy, we have studied a prototypical example for self-organized gradient formation in the model organism *Caenorhabditis elegans*. In particular, we have monitored in detail the formation of a concentration and mobility gradient of PIE-1 proteins as well as the partitioning behavior of vital organelles prior to the first, asymmetric cell division. Our data confirm the emergence of a concentration gradient of PIE-1 along the embryo’s anterior–posterior (AP) axis but they also reveal a previously unseen depletion zone near to the cell cortex that is not present for MEX-5 proteins. Time-resolved spatial diffusivity maps, acquired with SPIM-FCS, highlight the successive emergence of a mobility gradient of PIE-1 along the AP axis and suggest an almost linear decrease of fast diffusing PIE-1 proteins along the AP axis. Quantifying the subordinated dissemination of vital organelles prior to the first cell division, i.e. gradient formation on larger length scales, we find a significant asymmetry in the partitioning of the endoplasmic reticulum and mitochondria to the anterior and posterior part of the cell, respectively. Altogether, our spatiotemporally resolved data indicate that current one-dimensional model descriptions for gradient formation during *C. elegans* embryogenesis, i.e. a mere projection to the AP axis, might need an extension to a full three-dimensional description. Our data also advocate the use of lightsheet microscopy techniques to capture the actual three-dimensional nature of embryonic self-organization events.

Supplementary material for this article is available online

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(Some figures may appear in colour only in the online journal)
1. Introduction

Embryogenesis is a complex and fascinating self-organization phenomenon in which a full-grown organism develops out of a single fertilized oocyte (‘zygote’). Key steps of (early) embryogenesis are connected to major symmetry-breaking events, e.g. body axes formation or asymmetric cell divisions, that establish biochemically distinct organ precursors. In virtually all cases, the selection of distinct axes from a supposedly isotropic initial condition is connected to the dynamic formation and maintenance of protein or RNA gradients (see (Wieschaus 2016) and (Gonczy and Rose 2014) for recent reviews on the model organisms Drosophila melanogaster and Caenorhabditis elegans). Such gradients can be inherited or established already during oocyte formation, they can form in dependence on the location of sperm entry, they spontaneously emerge as Turing patterns, or they emanate as subordinate patterns to preexistent gradients. Hence, in all stages of development, an intimate and fairly complex interplay between biochemical and physical cues allocates spatial guidance for subsequent developmental stages.

Getting a quantitative understanding of the spatiotemporal dynamics during embryogenesis requires time-resolved three-dimensional imaging of specimen with a low phototoxicity to not impair the fragile organism. An advanced bioimaging technique that is particularly well suited for this task, is single-plane illumination microscopy (SPIM), also known as lightsheet microscopy (Keller et al 2008, Huisken et al 2004, Wu et al 2011, Krzic et al 2012, Huisken and Stainier 2009, Girkin and Carvalho 2018). SPIM relies on creating a thin lightsheet by a cylindrical lens or by scanning a line with a focussed laser beam in order to illuminate only a slice of a few μm thickness in the sample. Fluorescence from this illuminated slice is then detected in the perpendicular direction by a camera, i.e. all voxels in the illuminated sheet are imaged in parallel. A three-dimensional mapping of the probe is achieved by moving sample and lightsheet relative to each other. Due to only illuminating thin slices of the specimen, phototoxic effects are massively reduced (Keller and Stelzer 2008), making the method highly attractive for studying developmental self-organization.

Up to now a variety of SPIM implementations have been reported (see, for example, (Huisken and Stainier 2009), (Girkin and Carvalho 2018) for review) all of which allow for monitoring developing specimen in three dimensions over extended time scales. Moreover, SPIM also permits a non-invasive and rapid acquisition of diffusion maps, i.e. a laterally multiplexed diffusion measurement. To this end, fluorescence correlation spectroscopy (FCS) is performed in each of the several thousand pixels of a rapidly recorded SPIM image time series, i.e. local diffusion constants are revealed via the average autocorrelation time of fluorescence fluctuations within each pixel. SPIM-FCS has been shown to be a versatile tool for rapidly acquiring diffusion maps with high spatiotemporal resolution (Wohland et al 2010, Capoulade et al 2011, Krieger et al 2014) even within fragile developing embryos (Struntz and Weiss 2016). Being capable of monitoring three-dimensional concentrations of proteins as well as their local mobilities, lightsheet microscopy can retrieve important information on reaction–diffusion systems that drive gradient formation in developing organisms, e.g. in case of diffusion-mediated Turing patterns (Turing 1952). In very general terms, Turing patterns can arise in non-equilibrium systems when a uniform state of a non-linear, antagonistic reaction between two or more particle species becomes instable due to a poor mixing by the particles’ differing diffusion coefficients. An introduction to Turing patterns and related, more elaborate (‘Turing-like’) scenarios in the context of biology can be found, for example, in (Kondo and Miura 2010).

A particularly simple, yet generic model organism for studying developmental self-organization events and body axis formation, is the small transparent nematode Caenorhabditis elegans: C. elegans undergoes an invariant cell division scheme during embryogenesis and the zygote’s first cell division not only defines the head-to-tail body axis (antero–posterior, AP) but the emerging anterior and posterior daughter cells are the precursors for all somatic and germline cells of the developing worm (see (Gonczy and Rose 2014) for a comprehensive introduction). Symmetry breaking in this first embryonic cell division is governed by a Turing-like, advection-driven gradient formation of PAR proteins (Goehring et al 2011) whose membrane-bound fraction determines a larger anterior and a smaller posterior part of the zygote (marked by PAR-3/PAR-6 and PAR-1/PAR-2 proteins, respectively). Subordinated to the PAR gradient also other gradients emerge, e.g. for the proteins MEX-5 and PIE-1 that show an enrichment in the anterior and posterior part of the zygote, respectively (see figure 1). As a matter of fact, a posterior enrichment of PIE-1 in the zygote is necessary for determining the fate of the emerging posterior daughter cell as germline precursor (Reese et al 2000). Observation of MEX-5 or PIE-1 gradients therefore is an easy way to determine the anterior and posterior pole in unperturbed zygotes.

In fact, the enrichment of PIE-1 in the posterior part of the zygote is not only accompanied but actually driven by an anterior enrichment of MEX-5 (Reese et al 2000, Daniels et al 2009, Wu et al 2015). MEX-5 becomes phosphorylated at the posterior cell cortex by PAR-1, leading to an enhanced diffusional mobility of MEX-5 in the posterior part of the zygote (Daniels et al 2010, Griffin et al 2011). A uniformly distributed phosphatase switches MEX-5 back to a slow diffusive state (Griffin et al 2011), hence supporting a non-uniform steady-state distribution and a mobility gradient of MEX-5 that is subordinated to the primary PAR gradient. In terms of a quantitative model, the steady–state gradient formation of MEX-5 along the AP axis can be well described by a fairly simple reaction–diffusion system with a localized switching from a slow to a fast diffusing state (Daniels et al 2010, Griffin et al 2011). PIE-1 eventually attains an opposing concentration and mobility gradient via the same physical mechanism, induced by a MEX-5 dependent local switching of PIE-1 to a faster diffusive state (Daniels et al 2009, Wu et al 2015). As a consequence, PIE-1 becomes enriched in the posterior part of the zygote due to an elevated diffusional mobility in the anterior part.
So far, however, gradient formation of MEX-5 and PIE-1 was basically monitored only with respect to the AP axis, i.e. zygotes were imaged in their mid plane and only pixels near to the AP axis were considered for subsequent analyses. Consequently, also the accompanying reaction–diffusion models were only one-dimensional. Yet, given that the primary PAR gradient is defined only on the ellipsoidal cortex of the zygote, a mere projection to the AP axis might be an oversimplification of the whole process. Probing spatial dimensions perpendicular to the AP axis, e.g. with SPIM imaging, and potentially extending reaction–diffusion models beyond the one-dimensional formulation therefore is an important step towards a full understanding of this crucial gradient formation in the early development of C. elegans. In a similar fashion, SPIM can also be utilized to explore subordinated gradients on larger length scales, e.g. an asymmetric partitioning of organelles before the embryo undergoes its first cell division.

Following this rationale, we have used SPIM-based imaging to monitor PIE-1/MEX-5 gradient formation and asymmetric organelle partitioning in C. elegans zygotes in three dimensions over time. While the observed gradient formation of PIE-1 along the AP axis is consistent with previous reports, our data also highlight a significantly non-uniform distribution in the perpendicular direction, i.e. a successive depletion towards the cell cortex. In contrast, MEX-5 shows a uniform distribution perpendicular to the AP axis. These data suggest that a one-dimensional description of the PIE-1 gradient formation might be an over-simplification. Using SPIM-FCS, we also have probed the spatial variation of the diffusional mobility of PIE-1 during the crucial period of its gradient formation. While the overall trend of becoming faster in the anterior part of the zygote confirms previous observations, the obtained diffusion maps also highlight a considerable spatial heterogeneity of the diffusivity of PIE-1. These might be important ingredients for more elaborate reaction–diffusion models. Going beyond the emergence of protein gradients, we also quantified with three-dimensional SPIM imaging the subordinated patterning of vital organelles prior to the first cell division. Our data clearly show that the endoplasmic reticulum displays a significant and time-independent enrichment in the anterior part of the zygote whereas mitochondria rather are enriched in the posterior part. Therefore, somatic and germline precursor cells attain a significantly different organelle composition. Altogether, our data promote lightsheet microscopy as a versatile tool to quantitatively capture key physical quantities in three-dimensional embryonic self-organization events.

2. Materials and methods

2.1. Worm strains and sample preparation

In this study we used several C. elegans strains that were provided by the Caenorhabditis Genetics Center (CGC). In particular, JH1327 (axEx73 [pie-1p::pie-1::GFP + rol-6(su1006) + N2 genomic DNA]) (Reese et al. 2000) was used for imaging GFP-tagged PIE-1 and JH2840 (axls [nmy-2p::pgl-1::GFP::patr-1::nmy-2 3′UTR]; axIs1731 [pie-1p::mCherry::mex-5::pie-1 3′UTR + unc-119(+)]) (Gallo et al. 2010) for imaging mCherry-tagged MEX-5. For imaging the endoplasmic reticulum and mitochondria we used strains WH327 (ojls23 [pie-1p::GFP::C34B2.10]) (Poteryaev et al. 2005), expressing GFP-tagged SP12, and WH342 (ojls58[P-pie-1::spid-3::GFP + unc-119(+)]) (Poteryaev et al. 2005), expressing GFP-tagged SPD-3. In addition to the latter two we also used CAL0811 (unc-119(ed3) III; tIs37[pAA64-pie-1::mCherry::SP12; unc-119(+)] + ojls58[P-pie-1::spid-3::GFP + unc-119(+)]), expressing mCherry-tagged SP12 and GFP-tagged SPD-3, a kind gift of A. Kimura (NIG Japan). Worms were cultivated on nematode growth medium plates at 20.5°C (JH2840, WH327, WH342, CAL0811) or 24°C (JH1327) for proper gene expression. Measurements on organelle asymmetries (strains WH327, WH342 und CAL0811) were performed at 20.5°C, measurements on protein gradients were performed at 24°C. Therefore, worms of strain JH2840 were moved to 24°C two hours before starting the experiments so that embryos of strains JH1327 and JH2840 were measured under equivalent conditions. Sample preparation was done as described before (Fickentscher et al. 2013, Struntz and Weiss 2016, Fickentscher et al. 2016, Fickentscher and Weiss 2017, Fickentscher et al. 2018) by dissecting young adult worms to access freshly fertilized embryos. Embryos were placed in a 10–20 µl drop of M9 buffer on coverslips coated with Poly-L-Lysine (Biochrom, Berlin, Germany) before being placed on a custom-made sample holder in a water chamber at the aforementioned temperatures for imaging.

2.2. SPIM setup and image acquisition

Imaging was performed with a previously described, custom-made SPIM setup (see (Fickentscher et al. 2013, Struntz and Weiss 2016, Fickentscher et al. 2016, Struntz and Weiss 2018) for technical details). In brief, illumination of the specimen was done with a water-dipping objective (HCX APO L 10x/0.30 W U-V; Leica Microsystems) that focussed the lightsheet at a 45° tilt angle with respect to the horizontal sample plane. Fluorescence was collected by a second water-dipping objective (HCX APO L 40x/0.80 W U-V; Leica Microsystems) positioned perpendicular to the illumination lightsheet. The lightsheet had a minimal thickness (FWHM) of 1.4 ± 0.2 µm and 1.8 ± 0.2 µm for illumination with 491 nm and 561 nm as quantified by imaging a calibration sample (Alexa488 dye in 1% low-melting agarose; Carl Roth, Karlsruhe, Germany). Lateral and axial extensions of the point-spread function (PSF) were measured as described before (Struntz and Weiss 2018), yielding 510 nm and 1.4 µm for illumination with 491 nm and 561 nm as quantified by imaging a calibration sample (Alexa488 dye in 1% low-melting agarose; Carl Roth, Karlsruhe, Germany). Lateral and axial extensions of the point-spread function (PSF) were measured as described before (Struntz and Weiss 2018), yielding 510 nm and 1.4 µm for illumination with 491 nm and 561 nm. For imaging, the lightsheet was created by scanning a Gaussian beam through the sample twice per frame acquisition.

For time lapse measurements on the emergence of protein gradients, three-dimensional imaging was started shortly after fertilization (i.e. up to 10 min. before the pronuclear meeting) and was stopped in the four-cell state. Volumetric imaging relied on acquiring stacks of 51 images with an inter-layer distance of 1.4 µm within 6.8 s using a camera exposure time of 50 ms for each layer. Stack collection was repeated every 30 s.
Time lapse imaging on the establishment of organelle asymmetries relied on the same protocol but with a stack acquisition every 20 s. Laser power at the back aperture of the excitation objective was chosen between 0.2 mW and 2.3 mW which did not interfere with the embryo’s development. In all cases, 70–80 stacks of TIF files were collected for each embryo that were subject to a deeper analysis (see next subsection).

Slight variations in timing during worm dissection, sample preparation, and mounting resulted in a slight uncertainty in the precise developmental stage of individual embryos at the beginning of the measurement. Therefore, some zygotes had already progressed further to the pronuclear meeting than others. As a consequence, the number of individuals that contributed to the analysis varied for very early and also for very late times (see supplementary figure S1 (stacks.iop.org/JPhysD/53/295401/mmedia)). Nonetheless, the relevant period around the pronuclear meeting (for protein gradients) and around the first cell division (for organelle asymmetries) included basically all individuals.

2.3. Image analysis and data evaluation

First, all images within a stack were corrected for the lateral shift that is introduced by the 45° angle between the detection plane and the vertical movement of the sample stage (Fickentscher et al 2013, Fickentscher et al 2016, Fickentscher and Weiss 2017). After background subtraction (determined from pixels outside the embryo), lower and upper thresholds were defined to eliminate all pixels that do not belong to the cytoplasm, e.g. dim pronuclei and the first embryonic nucleus. These voxels were set to zero and did not enter the subsequent evaluation. As a consequence, a single contiguous ellipsoidal set of purely cytoplasmic voxels remained. Slight variations of this thresholding procedure did not alter the results.

To quantify apparent protein densities along the AP axis, the mean-intensity projection of all non-zero voxels onto each zygote’s long axis was calculated (see also supplementary figure S2 for the workflow). To this end, the mean of all pixel columns perpendicular to a chosen position x on the embryo’s long axis were assigned to this locus on the AP axis. For proper statistics, a minimum of 100 contributing voxels for each mean was demanded. To remove the influence of slightly different embryonic sizes, the AP axis was then rescaled to the unit interval for every embryo by dividing out the total length of the AP axis, $\ell$. Hence, positions on the rescaled AP axis were given by $\xi = x / \ell$ with $\xi = 0$ and $\xi = 1$ marking the anterior and posterior poles. To also remove variations in expression levels, mean intensities were normalized for each individual so that an integration over the AP axis ($0 \leq \xi \leq 1$) yielded unity. While individual profiles had comparable shapes even without, the data scatter was much lower after normalization (see supplementary figure S4). Approximating the local concentration of proteins in each voxel by the measured fluorescence intensity, this approach yields a proxy for the probability density function (PDF) of the respective protein along the rescaled AP axis in each individual. In order to align different embryos to a common time axis, we followed previous studies and set the pronuclear meeting as time point zero for all embryos. As a result of these spatial and temporal transformations, the imaging data of different embryos were suitable for an ensemble averaging.

To quantify protein distributions perpendicular to the AP axis, the corresponding fluorescence intensity was quantified in an radial fashion with respect to the central AP axis (see also supplementary figure S3 for the workflow). To this end, up to 18 concentric hollow cylinders with increasing radii $r = 1, 2, 3, \ldots \, \mu m$ were defined for each embryo and all nonzero voxels within the hollow cylinder’s volume were used to yield the average radial fluorescence. To guarantee enough statistics in the radial profile, a maximum radius of 15 $\mu m$ was considered since only very few embryos with a slightly different shape contributed to radii $r > 15 \, \mu m$. To allow for averaging over individuals and to highlight the potentially non-uniform shape of these radial profiles, radial fluorescence profiles were normalized by the respective maximum value at each time step.

In addition, we also used an ellipsoidal segmentation to better capture the zygote’s shape. To this end we applied tightly fitting ellipsoidal shells to the very same image stacks. Due to the embryo’s symmetry around the AP axis and its aspect ratio, we used rotational symmetric ellipsoids with a short axis $1 \, \mu m \leq a \leq 18 \, \mu m$ and a long axis $b = 2a$. As before, all nonzero voxels within an ellipsoidal shell, defined by the axis lengths $a$ and $a + 1 \mu m$, were used to determine the average fluorescence. Due to a too small number of contributing pixels, shells with $a < 3 \, \mu m$ and $a > 15 \, \mu m$ were not included in the analysis, i.e. normalization was performed with the maximum value in the range $3 \, \mu m \leq a \leq 15 \, \mu m$.

To further validate that non-uniform profiles are not simply due to slight variations in the geometry of individual embryos, we analyzed simulated embryos with the same scheme. Here, average embryonic ellipsoids with a random variation of the long axis (max. $\pm 6 \, \mu m$) were used. The background was set to zero and each voxel inside the ellipsoid was set to unity with a random variation of 10%. The resulting, ensemble-averaged radial profiles did not show any decrease towards the cortex (see supplementary figure S5) which supports our notion that the observed depletion of PIE-1 is not a mere geometric artifact.

In order to monitor the spatial partitioning of ER and mitochondria from SPIM-derived image stacks, we first determined for each embryo the center of mass and the principal radii of gyration of the ellipsoidal zygote. Since ER and mitochondria lie dense in the cytoplasm, scattering and absorption diminished the image quality for the embryonic hemisphere distal from the detection objective. Therefore we only considered imaging data from the proximal half of the embryo for the subsequent analysis. All remaining voxels were dissected into an anterior, middle, and posterior volume segment, equi-partitioned along the AP axis. In each volume segment we calculated the fluorescence density $\rho$, i.e. the ratio of the integrated fluorescence and the number of contributing voxels. Therefore, for an unbiased

\[
\theta = \frac{\sum_{i=1}^{n} x_i}{n}
\]
partitioning the density ratio of anterior and posterior volume segment, $\rho_A/\rho_P$, should fluctuate around unity as a function of time.

2.4. SPIM-FCS acquisition and data evaluation

SPIM-FCS measurements on embryos of strain JH1327 were performed as described before (Struntz and Weiss 2016). For each diffusion map, a sequence of 20,000 single images in the mid plane of an embryo was acquired at a rate of 828 frames per second (with exposure time 1 ms). We considered only the critical part of the gradient formation few minutes before, right at, and few minutes after the pronuclear meeting. Due to the short exposure times a higher laser power of 12.7 mW (measured at the back aperture of the excitation objective) had to be used to ensure a signal-to-noise ratio (SNR) that still allowed for a stable fitting of the derived autocorrelation curves in order to extract diffusion coefficients. A second measurement in the same embryo was performed at a higher laser power of 26 mW to compensate for a lower SNR due to bleaching from the previous acquisition. Due to the high laser power, not more than two measurements in a single embryo were possible without compromising the development. To further increase the SNR, a $2 \times 2$ on-chip binning on the camera sensor was used. Embryos were monitored until the 3–4 cell stage after measurements to ensure an unaltered development.

Data analysis of the image sequences was performed with Quickfit 3.0 (Beta, SVN: 4465) (Krieger and Langowski 2015). After background subtraction and bleaching compensation (Struntz and Weiss 2016) an additional software binning ($4 \times 4$) was used to ensure a sufficient quality of the autocorrelation function for fitting. For the resulting pixels, the autocorrelation function of the fluorescence signal was calculated separately and a fit based on normal diffusion of a single component in three dimensions (Singh et al 2013, Struntz and Weiss 2016) was used to derive the effective diffusion coefficient in the pixel location, resulting in a two-dimensional diffusion map for PIE-1. Here, only pixels showing the cytoplasmic part of the embryo were considered. By using an intensity-based threshold, pixels outside of the embryo were discarded, pixels within (pro)nuclei were masked manually. Fitting correlation data with more than one component or expressions for anomalous diffusion resulted in insignificant parameter estimations due to a too poor quality of the experimental autocorrelation data (underdetermined fit because of too many parameters). Therefore, only a single effective diffusion coefficient per pixel was retrieved by our SPIM-FCS data.

Similar to the evaluation of the intensity gradients described before, an average value of the effective diffusion coefficient along the AP axis was calculated by determining a mean value of all pixels in a column perpendicular to the long axis of the embryo. In order to average over multiple individuals, mean values of the diffusion coefficients along the AP axis have been calculated for an ensemble of at least eight measurements in different embryos for each time point (8–10 individuals for the indicated time points).

3. Results and discussion

3.1. Three-dimensional protein concentration gradients as seen with SPIM

As eluded to in the Introduction, MEX-5 and PIE-1 proteins are known to develop opposing concentration and mobility gradients until the first cell division. In fact, these gradients can, once established, even be used to distinguish the anterior from the posterior half of the zygote. Using SPIM imaging, we have first monitored the antipodal formation of protein concentration gradients in three dimensions over time (see figures 1(a)–(c) and 1(d)–(f) for representative mean intensity projections of image stacks for MEX-5 and PIE-1, respectively; representative projections to the xy- and yz-planes are shown in supplementary figure S6). A representative movie for the emergence of both gradients can also be found in the supplementary material. In agreement with earlier studies, our image series suggest a roughly uniform distribution for both proteins shortly after fertilization (figure 1(a) and (d)). At the pronuclear meeting this homogenous state is progressively tilted over to the anticipated steady-state gradients, i.e. MEX-5 becomes enriched in the anterior part of the zygote whereas PIE-1 is dominantly located in the posterior part (figure 1(b) and (e)). This non-uniform partitioning is maintained until the first two daughter cells have emerged (figures 1(c) and (f)).

Going beyond visual inspection of the images, we quantified gradient formation for an ensemble of embryos (not all embryos contributed to very early or very late time points, see Materials and Methods as well as supplementary figure S1). To support the ensemble averaging for embryos of slightly varying size, the actual length $\ell$ of the AP axis in each embryo was divided out, yielding rescaled positions $\xi = x/\ell$ in the unit interval. Approximating the local protein concentration by the measured fluorescence intensity, projecting all available voxel intensities onto the rescaled AP axis, and normalizing the integral intensity (see Materials and Methods for details) allowed us to extract the apparent average PDF, $p(\xi)$, of MEX-5 and PIE-1 along the AP axis (figures 1(g)–(i)). Notably, this approach only included voxels with a fluorescence intensity above a certain threshold to avoid an artificial averaging over empty voxels. Thresholding was chosen in such a way that for each individual in the ensemble of embryos only an ellipsoidal contiguous set of cytoplasmic voxels remained, i.e. all background voxels as well as pronuclei and the first embryonic nucleus were erased.

As a result, we observed significant differences in the emergence of both gradients (see figures 1(g)–(i)). While MEX-5 initially shows the anticipated, almost uniform distribution throughout the cell, the concentration of PIE-1 has a slight tilt towards the posterior end already at this early stage. This observation suggests that not only the action of MEX-5 is driving the emergence of a PIE-1 gradient. Supposedly, the distribution of PIE-1 proteins attains an initial tilt already by a direct or indirect interaction with the prime PAR gradient that is established before. At the pronuclear meeting, both protein gradients are firmly established, with a somewhat more pronounced asymmetry in the PIE-1 gradient. Until the first cell
division, both gradients were not seen to change grossly in strength or shape, i.e. the two-cell stage of the embryo basically quenches the non-uniform partition seen in the zygote at late stages. It is worth noting, however, that a slight decrease in \( p(\xi) \) close to the cell poles is observed for both proteins at all times. While this phenomenon is not significant at the anterior pole, i.e. standard deviations for \( p(\xi \leq 0.2) \) are so large that a depletion zone cannot be defined via local measures, the depletion of PIE-1 near to the posterior pole is fairly pronounced. We will come back to this observation when discussing our results for the radial part.

In order to quantify the temporal change in the steepness of both protein gradients along the AP axis we first determined the average value of \( p(\xi) \) in the anterior and posterior third of the zygote, i.e. we calculated \( R(t) = 3 \int_{0}^{1/3} p(\xi) d\xi \) for the anterior and \( R(t) = 3 \int_{2/3}^{1} p(\xi) d\xi \) for the posterior part. As can be seen in figures 2(a) and (b), stable gradients are formed around the pronuclear meeting \((t = 0)\), i.e. significant and stationary deviations from unity for the anterior and posterior third have emerged. As indicated already in the context of figure 1, the formation of a PIE-1 gradient seems to be slightly ahead of MEX-5. Since the uncertainty in determining \( t = 0 \) for each embryo via the pronuclear meeting is only \( \pm 15 \) s (i.e. half of the time between image stack acquisition), it is unlikely that the earlier emergence of a PIE-1 gradient is a complete artifact. Rather, our data indicate an initial pre-patterning of PIE-1, supposedly due to transient interactions with the prime PAR gradient that is established several minutes before the pronuclear meeting (Goehring et al 2011). In fact, this finding does not contradict the causal role of MEX-5 in firmly establishing the PIE-1 gradient, but our data suggest that a priming non-uniform initial condition might be established already before a visible MEX-5 gradient develops. Beyond the pronuclear meeting, i.e. after reaching a steady state for \( t \geq 0 \), the average anterior enrichment of MEX-5 is about 1.5 fold, whereas the average posterior enrichment of PIE-1 is about 2 fold. These ratios are somewhat less pronounced than in previous reports (Daniels et al 2009, Daniels et al 2010, Wu et al 2015, Wu et al 2018, Han et al 2018). We attribute this slight difference in the strength of the gradient to having included all voxels in three dimensions instead of focusing only on just one or few layers around the midplane, or only considering a stripe along the AP axis, both of which enhances the apparent gradient steepness when projecting it to the AP axis.

Since the first cell division in \( C. elegans \) is geometrically and biochemically asymmetric, we next sought for a measure to distinguish the cross-over position of the emerging gradients. To this end, we first smoothed the profiles \( p(\xi) \) for MEX-5 and PIE-1 by a moving average. Then, we searched for the steepest slope of the smoothed profile along the rescaled AP axis in the interval \( 0.1 \leq \xi \leq 0.9 \) to neglect boundary effects at the cell poles. About 4 minutes prior to the pronuclear meeting, i.e. when a marked gradient has emerged, a stable position \( \xi_0 \) for the steepest slope could be identified and followed until cytokinesis (figure 2(c)). Given the observation that the
crossover position of the prime PAR gradient, i.e. the crossover from PAR-3/6 to PAR-1/2 is significantly shifted away from the cell center towards the posterior pole (Goehring et al. 2011), we expected MEX-5 and PIE-1 to show values $\xi_0 > 1/2$. For MEX-5, however, the position of steepest slope was virtually always in the middle of the AP axis after the pronuclear meeting ($\xi_0 = 0.51 \pm 0.05$ for $t \geq 0$). In contrast, PIE-1 showed on average a marked shift towards the posterior pole ($\xi_0 = 0.61 \pm 0.07$ for $t \geq 0$), in agreement with the anticipated retracing of the PAR gradient. A two-sample Kolmogorov–Smirnov test confirmed different $\xi_0$ for MEX-5 and PIE-1 in the domain $t \geq 0$ s at a significance level below 1%. This finding further supports the notion that PIE-1 might align its gradient not solely via MEX-5 but also via direct interactions with PAR proteins.

Exploiting the three-dimensional nature of our SPIM data, we next probed to which extent MEX-5 and PIE-1 concentrations are uniform perpendicular to the AP axis. To this end, we quantified the normalized radial intensity distribution, $F(r)$ of both proteins with respect to the AP axis (figures 3(a)–(c)) and via segmenting the embryo with ellipsoidal shells (figures 3(d)–(f)); see Materials and Methods for technical details. As a result, we found that MEX-5 indeed features a homogenous distribution in the radial direction at all times with only mild excursions (consistent with a constant within the indicated standard deviation). Hence, all information on MEX-5 gradient formation is contained in the projection onto the AP axis. For PIE-1, however, we observed a significant depletion near to the cell cortex that was noticeable already 5–10 $\mu$m away from the cell surface. This radial depletion of PIE-1 at the cell surface is quite pronounced before the pronuclear meeting and is diminished in later stages (figure 3). To probe the significance of this observation we employed a two-sample Kolmogorov–Smirnov test (5% significance level). As a result, we found that both, $F(r)$ and $F(a)$ were significantly different for PIE-1 for all measured times when comparing positions $r, a = 3 \mu$m to $r, a = 15 \mu$m. Therefore, the observed depletion of PIE-1 is indeed significant. Yet, as expected already by visual inspection, the softening of PIE-1 depletion upon approaching the pronuclear meeting lead to a more differential rating. Comparison of PIE-1 to MEX-5 at $r, a = 15 \mu$m indicated a significant difference between the two protein species for almost all times when using $F(a)$, whereas the radial analysis negated a significant difference at many time points around the pronuclear meeting.

The radial depletion of PIE-1 within a boundary layer of about 5 $\mu$m beneath the cell surface matches well to the apparent depletion of PIE-1 at the posterior pole (cf figure 1): A depletion along the AP axis is seen in the range $\xi < 0.9$ which translates into a roughly 5 $\mu$m wide posterior depletion zone when considering the typical length $\ell = 50 \mu$m of the embryo’s long axis. It is conceivable that mere sterical exclusion by the actomyosin cortex is responsible for this depletion. Yet, MEX-5 is not affected in the same way, suggesting that it is not simply the dense cortical actomyosin network that is responsible for the observed depletion. Supposedly, interactions with the cytoplasmic pools of MEX-5 and/or PAR proteins, suggested to be mandatory for gradient formation (Griffin et al. 2011), are also contributing to the depletion zone. In any case, our findings suggest that current one-dimensional reaction–diffusion models (Wu et al. 2018) might require an update to three dimensions to capture all relevant processes during gradient formation. Spatial inhomogeneities in the cytoplasm could also be considered in such a model refinement, which stimulated us to also probe the local protein mobility of PIE-1 via SPIM-FCS.

3.2. Diffusion maps of PIE-1 during gradient formation as seen with SPIM-FCS

Given that PIE-1 showed a somewhat more complex partitioning behavior than MEX-5, and bearing in mind that
Figure 3. Upper panel: radial distributions of the normalized intensity, $F(r)$, of MEX-5 (black) and PIE-1 (red) at three different times until the pronuclear meeting ($t = 0$). Lower panel: corresponding normalized intensity, $F(a)$, obtained via segmenting zygotes with ellipsoidal shells (short and long axes having lengths $a$ and $2a$, respectively). Standard deviations are indicated by shaded regions. While MEX-5 shows an almost uniform radial distribution at all times (in both evaluation approaches), PIE-1 shows a marked depletion within a distance of 5 $\mu$m to the cell surface at early time points. This depletion phenomenon progressively softens while the gradient along the AP axis is established (until the pronuclear meeting). See main text for discussion. Averaging involved $n = 5$ ($t = -420$ s), $n = 11$ ($t = -210$ s), and $n = 12$ ($t = 0$ s) embryos (see also supplementary figure S1).

Figure 4. (a) Representative individual autocorrelation curves from the anterior (black circles) and posterior (red squares) part of the zygote. A markedly larger diffusional time scale $\tau_D \sim 1/D$ is seen for the latter. Despite a strong scatter in the data, fitting with an expression for a single diffusing species was stable (full lines). Fitting with more elaborate models, however, yielded only instable parameter estimates; see also main text for discussion. (b) Effective diffusion maps of PIE-1 in the embryo mid-plane before, at, and after the pronuclear meeting (from top) indicate some spatial diffusion heterogeneity and an increasing mobility gradient along the AP axis. Regions outside of the cytoplasm and regions including pronuclei and the first nucleus have been masked. (c) The ensemble-averaged projection of diffusion maps onto the AP axis ($-12 \mu m \leq x \leq 25 \mu m$) highlights a mobility gradient, i.e. a decreasing effective diffusion coefficient in the anterior–posterior direction. This mobility gradient is enhanced until and beyond the pronuclear meeting (see color-coding black to blue to red). Shaded regions indicate the embryo ensemble’s standard deviation. Inset: translating the effective diffusion coefficient in the zygote to the fraction $f$ of a fast component, in the presence of a slow component with fraction $1 - f$, indicates a roughly linear change from a majority of fast proteins in the anterior to a vastly dominating majority of slow proteins in the posterior. See main text for details of how to estimate $f$.

PIE-1 gradient formation has been implicated to rely on a spatial diffusion heterogeneity, we employed SPIM-FCS to acquire spatial diffusion maps for PIE-1 (see Materials and Methods for details). In particular, we focused on three important periods during the emergence of the gradient: a few minutes before, right at, and few minutes after the pronuclear meeting. This covers the critical period of PIE-1 gradient formation i.e. shortly after fertilization (before gradient formation), at the pronuclear meeting (almost fully established gradient), and in the one-cell stage (firmly stabilized gradient). Since technical limitations of current sCMOS cameras render SPIM-FCS curves too noisy to allow for a significant two-component or even anomalous diffusion fit (see (Struntz and Weiss 2016) for discussion), we fitted all autocorrelation curves with only a single diffusive component (see figure 4(a) for representative examples). As a result, we obtained a spatial map of effective diffusion constants, $D_{\text{eff}}$, which is somewhat less informative than smoother single-point FCS.
Subsequently and persists well beyond the pronuclear meeting, a gradient of effective diffusion coefficients is stabilized at the outmost anterior pole. About 49% of the embryos exhibited a rough estimate for the fraction $f$ of PIE-1 proteins based on the measured effective diffusion coefficients along the AP axis (Daniels et al 2009). To still arrive at an estimate how the superposition of two diffusing species would be seen when fitting with a single-component FCS curve, given $f = (1 + \tau_D/\tau)$, yields a proxy for how the superposition of two diffusing species would be seen when fitting with a single-component FCS curve. Inserting $\tau_D = \tau_D/(4D_{\text{eff}})$, $\tau = \tau_D/(4D_f)$, and $\tau_D = \tau_D/(4D_s)$ eventually yields $f = (1 + D_f/D_{\text{eff}})(D_s - D_{\text{eff}})/(2(D_f + D_s))$. For simplicity, we have used for this consideration only the dominant in-plane autocorrelation decay, i.e. diffusion in two dimensions. Assuming $D_f = 10 \mu m^2/s$ and $D_s = 1 \mu m^2/s$, consistent with previous two-component FCS measurements (Daniels et al 2009), therefore yielded a rough estimate for the fraction $f$ of fast diffusing PIE-1 proteins based on the measured effective diffusion maps.

Three representative examples for diffusion maps taken in the aforementioned periods of PIE-1 gradient formation are shown in figure 4(b). Before the pronuclear meeting, diffusion coefficients are mostly uniform with values in the range $1 \mu m^2 s^{-1} \leq D_{\text{eff}} \leq 2 \mu m^2 s^{-1}$. Some random heterogeneities are seen throughout the embryo with a tendency for elevated diffusion constants at the outmost anterior pole. About the pronuclear meeting a significantly higher portion of pixels with larger diffusion constants in the range $D_{\text{eff}} \geq 5 \mu m^2 s^{-1}$ has emerged in the anterior part of the zygote. This distinct gradient of effective diffusion coefficients is stabilized subsequently and persists well beyond the pronuclear meeting, until the first cell division.

For a better visualization and for a proper comparison to previous work, we have also compiled ensemble-averaged effective diffusion coefficients along the AP axis for all measurements (figure 4(c)). These data indicate an elevated mean value of the effective diffusion coefficient on the anterior side already before the pronuclear meeting. This initially slight gradient increases considerably for later stages, highlighting a more rapid diffusion of PIE-1 in the anterior part of the zygote, in agreement with earlier observations based on single-spot FCS, fluorescence recovery after photobleaching, and single-particle tracking (Daniels et al 2009, Wu et al 2015, Wu et al 2018). As compared to these earlier experiments, SPIM-FCS allowed us to acquire full spatial diffusion maps along the entire AP axis as a function of developmental time, which was hardly possible with previous approaches. It is worth noting at this point that SPIM-FCS can, in principle, also probe diffusion on different length scales by varying the extent of software-binning of pixels (Bag et al 2012, Veerapathiran and Wohland 2018). In our case, however, a trade-off between the desired spatial resolution of diffusion maps and the poor signal-to-noise ratio of autocorrelation curves did not support this approach.

Translating from an effective diffusion coefficient to fractions of fast and slow diffusing species (as outlined above) reveals an almost linear reduction of the fast component from the anterior to the posterior pole beyond the pronuclear meeting (figure 4(c), inset). This finding suggests that the mobility gradient is much more smeared out than expected from the rather steep gradient in the concentration profiles (figure 1). Along the radial direction, i.e. perpendicular to the AP axis, we did not note discernible variations of the diffusional behavior of PIE-1, indicating that no major diffusion barriers are responsible for the depletion zone seen before (cf figure 3). Hence, for the mobility part of a reaction diffusion model the projection onto the AP axis alone appears sufficient.

Figure 5. Representative maximum projections of SPIM image stacks for (a) and (b) ER and (c) and (d) mitochondria at late stages of the zygote (a) and (c) and in the two-cell stage (b) and (d). A biased partitioning of both organelles to the anterior and posterior part is visible. (e) Quantifying the effect via the ratio of the fluorescence densities in the anterior and posterior part, $\rho_A/\rho_P$, indicates a clear anterior enrichment of the ER at all times (black line) whereas mitochondria rather accumulate in the posterior part (red line). For comparison, an unbiased partitioning ($\rho_A = \rho_P$) is indicated by the black dashed line. Shaded regions are standard deviations from the ensemble of embryos. For convenience, times are expressed here relative to the first cell division ($t_{CD}$) in which organelles become irreversibly confined in daughter cells. Scale bars: 10 µm. Please note that the full ensemble of embryos contributed in the range $-200 s \leq t \leq 200 s$ whereas less individuals were available at other times (cf supplementary figure S1).

measurements that have revealed the co-existence of a fast and a slow fraction throughout the zygote, yet with a varying fraction $f$ of the fast pool along the AP axis (Daniels et al 2009). To still arrive at an estimate how the measured effective diffusion constants emerge from a superposition of a fast and a slow diffusing pool of proteins, we used the following consideration: let us assume two components with diffusion constants $D_f$ and $D_s < D_f$, having fractions $f$ and $1 - f$, respectively. The decay half-time $\tau_D$ of a two-component FCS curve, given by $f/(1 + \tau_D/\tau) + (1 - f)/(1 + \tau_D/\tau) = 1/2$, yields a proxy for how the superposition of two diffusing species would be seen when fitting with a single-component FCS curve. Inserting $\tau_D = \tau_D/(4D_{\text{eff}})$, $\tau = \tau_D/(4D_f)$, and $\tau_D = \tau_D/(4D_s)$ eventually yields $f = (1 + D_f/D_{\text{eff}})(D_s - D_{\text{eff}})/(2(D_f + D_s))$. For simplicity, we have used for this consideration only the dominant in-plane autocorrelation decay, i.e. diffusion in two dimensions. Assuming $D_f = 10 \mu m^2/s$ and $D_s = 1 \mu m^2/s$, consistent with previous two-component FCS measurements (Daniels et al 2009), therefore yielded a rough estimate for the fraction $f$ of fast diffusing PIE-1 proteins based on the measured effective diffusion maps.

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3.3. Asymmetric partitioning of organelles

Going beyond the formation of protein gradients, we also used SPIM imaging to explore downstream gradients on larger length scales. In particular we probed whether partitioning of organelles into the emerging daughter cells also follows a gradient or if this is rather a random process. Earlier qualitative studies indicated that at least mitochondria might show a biased partitioning in *C. elegans* and another nematode (Badrinath and White 2003) during the first cell division. To explore this in more detail, we imaged not only mitochondria but also the endoplasmic reticulum (ER) with SPIM (see Materials and Methods). In fact, earlier studies also suggested an unequal partitioning of the ER during the first cell division (Poteryaev et al 2005). As can be seen from the representative maximum intensity projections in figure 5, both organelles exhibit a preference for one side of the zygote (see supplementary figure S7 for individual image planes). While the ER is more enriched in the anterior part, mitochondria rather accumulate in the posterior part. To quantify this effect, we determined for both organelles the ratio of the fluorescence densities in the anterior and posterior part, $\rho_A/\rho_P$ (see Materials and Methods for details). If there was no bias, one would expect $\rho_A/\rho_P \approx 1$ for an ensemble of embryos. However, we observed a pronounced deviation from this expectation for both organelles, i.e. using the standard deviation as the measure of uncertainty, the mean for both organelles deviated significantly from unity (figure 5). While the ER showed a time-independent (constant) shift towards the anterior part of the zygote, mitochondria were enriched in the posterior part. No change in this partitioning was seen until and beyond the first cell division.

It is worth noting at this point, that measurements for protein gradient formation and organelle partitioning had to be performed at slightly different temperatures (see Materials and Methods). Thus, if we were to compare the dynamic evolution of both, an Arrhenius scaling of developmental time scales would have to be considered (Fickentscher et al 2016, Fickentscher et al 2018). However, organelle partitioning was seen to be time-independent around the first cell division. The length of this period may certainly vary with temperatures, yet the partition asymmetry itself is unlikely to be affected.

At first glance, the roughly 10% difference in the anterior and posterior fluorescence densities might not appear as a large effect. However, transferring this to all contributing voxels and bearing in mind the volume ratio of the emerging daughter cells in the first cell division ($V_A/V_P \approx 1.4$ (Fickentscher and Weiss 2017)), a significantly larger portion of the ER is partitioned into the larger, anterior daughter cell that is the precursor for all somatic cells. Conversely, the smaller, posterior daughter cell (the germline precursor) inherits a significantly larger mass of mitochondria. It is tempting to speculate that this unequal partitioning also triggers differences in the subsequent development of these two precursor cells for the somatic and germline lineages.

Altogether, these data and the insights obtained in the preceding subsections demonstrate that lightsheet microscopy is a versatile tool to quantitatively capture key physical quantities in three-dimensional embryonic self-organization events, from the level of protein concentrations and mobilities up to the mesoscopic partitioning of organelles.

4. Conclusion

Using lightsheet microscopy (SPIM) we have quantified the emergence of protein concentration and mobility gradients as well as a non-uniform partitioning of vital organelles during the early embryogenesis of *C. elegans*. Unlike previous quantifications that were based on single planes in the embryo, SPIM allowed us to explore three-dimensional properties of these gradients over time. In particular, we could not only confirm the general concentration gradients of two critical proteins, MEX-5 and PIE-1, along the AP-axes but our data also revealed a so far unreported depletion of PIE-1 in the vicinity of the cell cortex. Our spatially resolved diffusion measurements of the mobility of PIE-1 via SPIM-FCS are in accordance with previous results but also highlight the spatiotemporal evolution of the emerging mobility gradient in previously unresolved detail. In combination, our data on PIE-1 indicate that current model descriptions for the emergence of a concentration and mobility gradient might need an extension beyond a mere one-dimensional projection onto the AP axis. Moreover, additional regulation loops beyond the MEX-5 driven change of the diffusion coefficient of PIE-1 might have to be considered.

Due to the ability to perform gentle long-term three-dimensional imaging of early *C. elegans* embryos with SPIM, we were also able to reveal a significant and antipodal asymmetry in the partitioning of the endoplasmic reticulum and mitochondria during the first cell division. These data highlight important cell-biological consequences of the priming protein gradients for the subsequent development of the embryo since the first two daughter cells are precursors for all somatic and germline cells, respectively.

In summary, our data demonstrate that imaging and multiplexed spatial diffusion measurements with lightsheet microscopy are well suited to explore the three-dimensional biology of fragile embryos over time, even in crucial stages of their development.

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