Methods

Quantitative and dynamic cell polarity tracking in plant cells

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Summary

- Quantitative information on the spatiotemporal distribution of polarised proteins is central for understanding cell-fate determination, yet collecting sufficient data for statistical analysis is difficult to accomplish with manual measurements.
- Here we present Polarity Measurement (POME), a semi-automated pipeline for the quantification of cell polarity and demonstrate its application to a variety of developmental contexts.
- POME analysis reveals that, during asymmetric cell divisions in the Arabidopsis thaliana stomatal lineage, polarity proteins BASL and BRXL2 are more asynchronous and less mutually dependent than previously thought. A similar analysis of the linearly arrayed stomatal lineage of Brachypodium distachyon revealed that the MAPKKK BdYDA1 is segregated and polarised following asymmetrical divisions.
- Our results demonstrate that POME is a versatile tool, which by itself or combined with tissue-level studies and advanced microscopy techniques can help to uncover new mechanisms of cell polarity.

Introduction

Cell polarity is the central mechanism responsible for organising a cell into subdomains with specialised functions. The subcellular enrichment of polarity proteins enables complex processes like cell migration, directional long-range signal transduction, cell growth anisotropy and asymmetrical cell division (ACD) (Drubin & Nelson, 1996; Muroyama & Bergmann, 2019). Due to the sessile lifestyle of plants, cell polarity is particularly important for the proper execution of developmental and physiological programmes. Plants need to adjust their development based on extrinsic cues and the lack of cell migration puts greater emphasis on the orientation of ACDs and differential cell expansion for tissue patterning.

Although cell polarity can be manifested in the distribution of many components, including organelles, RNAs and metabolites, proteins polarly distributed at the cell cortex play especially prominent roles in plants. These ‘polarity proteins’ may be integral or peripheral membrane components and are often encoded only in plant genomes. Their distribution defines cellular and tissue-level axes, and they direct the development of major body axes, tissue layers and the distribution of specialised cell types within a tissue (Muroyama & Bergmann, 2019). For example, in the stomatal lineage of the Arabidopsis leaf epidermis, polarly localised BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) and the BREVIS RADIX family (BRXf) are required to orient ACDs and ensure that the daughters of such divisions take on different fates (Dong et al., 2009; Rowe et al., 2019).

Many other proteins exhibiting polarised distributions in plants have been identified over the past decade, but our understanding of the mechanisms that control their distribution is still in its infancy (Dong et al., 2009; Scacchi et al., 2009; Zourelidou et al., 2009; Houbaert et al., 2018; Marhava et al., 2018; Denninger et al., 2019; Yoshida et al., 2019; Tan et al., 2020; van Dop et al., 2020; Zhang et al., 2020). Quantifying the distribution of polar proteins in time and space is a necessary first step in understanding how cell polarity is formed and coordinated with neighbouring or daughter cells. Comparing the polar domain size of different polarity proteins can help infer how protein complexes are assembled, while the temporal dynamics of the protein abundance along the membrane can reveal trafficking and post-translational regulatory mechanisms.

In animal systems, quantification of polarity proteins has already challenged old paradigms and revealed unforeseen phenomena. For example, PAR proteins in animal cells have been known for nearly 30 yr, yet a recent 3D image segmentation and quantitative analysis of PAR proteins in the C. elegans embryo germline revealed that PAR polarity domain size does not scale with cell size and this intrinsic property regulates the timing of cell-fate transition (Hubatsch et al., 2019).

Similar quantitative analyses in plants would help to identify critical players and novel mechanisms in the establishment of cell

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polarity. However, most studies so far have relied heavily on tedious visual inspection and remain qualitative (Wisniewska et al., 2006; Dong et al., 2009; Scacchi et al., 2009; Zhang et al., 2016; Marhava et al., 2018; Rowe et al., 2019; Yoshida et al., 2019; van Dop et al., 2020). Early efforts to define ‘polarity indices’ fall roughly into two categories: (i) those that compare protein abundance between the peak of the polarity ‘crescent’ and the opposite side of the cell; and (ii) those that measure the relative size of the crescent. Neither of these two types of polarity indices captures the full picture of protein distribution. Polarity indices that only compare protein abundance between poles may be insufficient to distinguish between the subtle contributions of the multitude of processes that shape the distribution (Marhavy et al., 2014; Zhang et al., 2015; Langowski et al., 2016; Houbaert et al., 2018; Denninger et al., 2019). Conversely, polarity indices that consider only the width of the crescent (Zhang et al., 2015) and do not compare protein abundance at the poles, are also insufficient to fully describe polarisation, as we will show in this work.

Here, we present Polarity Measurement (POME), an image analysis pipeline designed to measure polarity in plant cells from confocal fluorescence images. Our tool converts the measurement of individual cells into data sets that can be interrogated with a variety of statistical tools. We demonstrate the use of POME to systematically quantify the polarity of BASL and BRXL2 over time in the Arabidopsis stomatal lineage. Based on these detailed measurements, we modify previous models for how these proteins establish and maintain polarity in stomatal lineage cells. We also demonstrate how POME can quantify aspects of asymmetric protein distributions in different tissues and organisms and again reveal that previous models for polarity establishment must be reconsidered. Finally, to facilitate community access and implementation of POME, we provide our pipeline and documentation in an accessible format.

Materials and Methods

Plant material

All Arabidopsis lines used in this study were generated in the Col-0 background. Construction of all polarity reporter lines (pBRXL2::BRXL2-YFP pML1::mCherry-RCI2A, pBASL::YFP-BASL pML1::mCherry-RCI2A, pBASL::Myr-BRX-YFP pML1::mCherry-RCI2A brx1, pBRXL2::BRXL2-YFP pML1::mCherry-RCI2A basl-2, pPIN2::PIN2-GFP) have been reported elsewhere (Wisniewska et al., 2006; Rowe et al., 2019). The Brachypodium BdYDA1 reporter line pBdYDA1::BdYDA1-YFP was generated in the Bd21-3 ectotype as described elsewhere (Abrash et al., 2018).

Plant growth conditions

All Arabidopsis seeds were surface sterilised by bleach or 75% ethanol and stratified for 2 d. After stratification, seedlings were vertically grown on half-strength Murashige and Skoog (½MS) medium for 3–6 d under long-day conditions (16 h : 8 h, light : dark, at 22°C).

Microscopy and image acquisition

All imaging experiments for Arabidopsis were performed on a Leica SP5 confocal microscope with HyD detectors using x25 NA0.95 and x40 NA1.1 water objectives with image size 1024 × 1024 and digital zoom from ×1 to ×2.5. For time-lapse experiments, 3-d post germination (dpg) seedlings were mounted in a custom imaging chamber filled with ½MS solution (Davies & Bergmann, 2014; Bringmann & Bergmann, 2017; Simmons et al., 2019). Laser settings for each reporter, except the membrane marker (pML1::mCherry-RCI2A or PI), were adjusted to avoid over-saturation. For the time-lapse experiments reported in this study, 30 or 40 min intervals between each image stack capture were used.

Images of the BdYDA1 reporter in developing Brachypodium leaves were reanalysed from the same set of raw images reported in Fig. 3 of Abrash et al. (2018). Images of PAR dynamics in C. elegans embryos were reanalysed from the raw images of supplementary video 2 reported in Hubatsch et al. (2019).

POME analysis of fluorescence images

All raw fluorescence image Z-stacks were projected with Sum Slices in FIJI unless noted otherwise. For all time-lapse images, drift was corrected using the CORRECT 3D DRIFT plugin (Parslow et al., 2014). These time-lapse images were then split into individual time frames and treated as still images for analysis afterwards. Each individual cell of interest was then examined for manual correction before quantification, when regions containing interfering vesicles (images with FM4-64 staining) or nucleus (images of BASL reporter) were manually removed. When running POME (Supporting Information Methods S1) on a selected cell, the settings were adjusted per experiment. Information on how to determine POME settings and examples of POME data analysis are described in detail in the POME user guide files (Notes S1, S2) accompanying this manuscript. The results of POME measurement were then imported, summarised and analysed in RSTUDIO.

Fitting the polarity protein distribution along the cell membrane to a Gaussian model

For polarity proteins in Arabidopsis and Brachypodium stomatal lineage, the mean fluorescence intensity of the polarity protein reporter along the cell membrane was fitted to a Gaussian model (Eqn 1) by nonlinear regression with the nls function from the stats package (R Core Team, 2020) in RSTUDIO. Key parameters of this regression model, including SD σ, centre µ, amplitude α and baseline value β, were estimated using the formula:

\[ f(x) = ae^{-\frac{1}{2}(\frac{x-\mu}{\sigma})^2} + \beta \]

Eqn 1

Generating polarity classification matrices

To determine if the parameters (σ, α and β) estimated by POME were sufficient to distinguish between polarised (BRXL2 in stomatal lineage ground cells and BASL) and depolarised (BRXL2 in...
guard mother cells and Myr-BRX) markers, we used measurements from 80 cells (20 cells per marker) to train a binary logistic regression model using the glm function from the STATS package in RStudio. To visualise each parameter’s ability to classify polarity, a receiver operating characteristic (ROC) plot was generated with the roc function from the RROC package (Robin et al., 2011). The resulting plot (Fig. 1h) suggest that σ alone could successfully discriminate between polarised and depolarised markers. Next, a simpler binary logistic regression model was built using only σ (Fig. 1i) to determine a suitable cut-off value. To calculate the error rate of the polarity ~ σ logistic regression model, a 10-fold cross-validation prediction error was calculated with the training dataset with the cv.glm function in the boot package (Davison & Hinkley, 1997), where an average reporting error of 9.2% was reported.

For the support vector machine (SVM) model, the parameters from the same 80 cells in the binary logistic regression model were used as training data. To remove imaging setting and reporter basal expression bias, a normalised amplitude α was calculated by dividing the amplitude α by the mean fluorescence intensity of all angles. The SVM model was then built with the svm function in the e1071 package (Meyer et al., 2019). Next, linear coefficients were extracted from the model to calculate the slope (3.672) and intercept (0.069) of the decision boundary (Fig. S2b). We can then define an SVM polarity index as the distance to the SVM model decision boundary calculated with the formula:

\[
\text{SVM polarity index} = \frac{-\log_\sigma(\sigma) + 3.672\sigma + 0.069}{\sqrt{(-1)^2 + (3.672)^2}}
\]

Eqn 2

A positive SVM polarity index indicates a polarised cell, while a negative SVM polarity index indicates a depolarised cell. Higher SVM polarity index values indicate higher polarisation.

Statistical analysis

All statistical analyses were performed in RStudio. Unpaired Mann–Whitney U-tests were conducted to compare two data samples with the compare_means function from the ggpubr package (Kassambara, 2020).

Results and Discussion

**POME**: a semi-automated tool to quantify cortical protein polarity

The Arabidopsis stomatal lineage is a powerful model for genetic analysis of asymmetrical divisions, but mechanistic dissection of cell polarity has been challenging. BASL and BRXL2 polarity (demonstrated with BRX and BRXL2) is required for ACDs (Dong et al., 2009; Rowe et al., 2019), but how these proteins initiate and maintain polarisation is not understood. The primary challenge hindering quantitative analysis in the stomatal lineage is the rare and transient nature of cells undergoing ACDs, and demands the imaging of a large number of samples to discern statistically significant differences between genotypes or conditions. Additionally, cell shapes in the stomatal lineage are irregular and variable, which can make drawing conclusions from visual inspection difficult and adds uncertainty to manual measurements.

To reduce the time required for quantification and to collect shape-agnostic measurements, we developed POME, a pipeline composed of an intuitive Fiji macro and an R script that automatically extracted cortical polarity information from user-defined cells. POME requires confocal images of a cell with two different channels. One of these channels must contain a uniform cell outline marker (e.g. integral plasma membrane reporter, FM 4-64 or propidium iodide). The macro then quantifies the fluorescence of polar proteins in the second channel at different angles (Fig. 1a–d). This simplification provides comparable results that are independent of cell shape. Users can specify the number of measurements (i.e. angles) along the membrane.

The macro calculates an angle of polarisation with respect to the field of view after extracting the centroid and the centre of mass of the polar protein in the measured cell from the outline marker and the polar protein channels, respectively. A line from the centroid to the centre of mass defines the 0° and 180° angles used for reporting results (Fig. 1b). Further analysis of these angles of polarisation could reveal the existence of tissue-level coordination, as has been shown by previous studies (Bringmann & Bergmann, 2017; Mansfield et al., 2018).

Initially, we used POME to quantify the distribution of BRXL2 in stomatal lineage ground cells (SLGCs) and found that its protein abundance at the membrane is well represented by a Gaussian model (Fig. 1c). This allowed us to describe the BRXL2 localisation with three parameters: SD, σ, amplitude (α) and baseline intensity (β) (Fig. 1c).

To evaluate the capacity of POME to distinguish among degrees of polarity, we applied it to three additional cases: BASL (polarised), BRXL2 in guard mother cells (GMCs, depolarised) and myristoylated BRX (Myr-BRX, depolarised) (Figs S1f,S1). These cases present a variety of challenges to polarity quantification, including signal interference from the nucleus (BASL), weak expression causing a low signal-to-noise ratio (depolarised BRXL2 in GMCs) and signals from abutting membranes in two neighbouring cells interfering with each other signals (myr-BRX). We selected 20 cells for each marker and measured their distribution along the cell periphery. The distributions of these markers were different, but they can all be described by a Gaussian model (Fig. 1f). The SD, amplitude and baseline intensity (Fig. 1g) for BASL and the polarised BRXL2 were statistically indistinguishable (Fig. S2a). The distribution of the depolarised (cytosolic) BRXL2 and Myr-BRX were clearly different from each other and from the distribution of the polarised BASL and BRXL2. Note that the SD of depolarised BRXL2 and Myr-BRX were statistically indistinguishable, but their amplitudes and baselines were not, reflecting differences in fluorescence levels and possibly protein abundance (Fig. 1g).

Next, we compared the SD, amplitude and baseline intensity between our polarised and depolarised datasets using binary
logistic regression and generated a ROC curve for each parameter to test its ability to classify polarity (Fig. 1h), where the closer the curve is to the upper left corner on the ROC plot, the higher the predictive power of the parameter. These results suggested that SD alone is predictive of polarity. Furthermore, SD is nearly independent of the expression level of the marker and the imaging setting, making it a reliable polarity classifier. To determine the cut-off value for polarity classification using SD as the sole classifier, we plotted the sensitive (true positive rate) and specificity (true negative rate) for each SD cut-off value. We found...
that a cut-off of 42° can correctly discriminate between a polarised and a depolarised marker with a 95% classification sensitivity and 83% specificity, while a cut-off of 60° achieved 100% classification sensitivity and 78% specificity (Fig. 1i,j). Alternatively, an SVM algorithm can be used to generate a polarity classification model with 100% classification sensitivity and 100% specificity using the SD and normalised amplitude ($\alpha'$ amplitude divided by mean fluorescence intensity) (Fig. S2b,c).

Characterising the dynamics of polarity proteins in the stomatal lineage

We implemented POME to analyse the dynamics of BASL and BRXf distribution. Biochemical studies indicated that BASL and BRXf interact, while genetic analysis indicated that they require each other to remain polarised (Rowe et al., 2019). BASL and BRXf have been shown to occupy the same general region of the cell cortex, but the evidence is limited to single time point images and qualitative interpretation. As a result, it is unclear if their polarity domains always overlap or whether one protein polarises first and then recruits the other.

To address this question, we first measured the distribution of BRXL2 before and after an ACD, from the initial formation of its polar crescent to its complete dissociation from the membrane (Fig. 2a). We fitted the fluorescence measurements of each time frame to a Gaussian model and extracted the SD, amplitude, and baseline ($\beta$). BRXL2 fluorescence intensity of each measured pixel ($\sigma$) and the average BRXL2 fluorescence intensity of all the measured pixels at a given angle ($\alpha$) are represented by dots coloured by their fluorescence intensity. (iv) Reconstruction and visualisation of cell polarity from POME measurements. The cell centroid is marked with ‘+’. BRXL2 fluorescence intensity along the membrane. A line is drawn from the cell centroid (white dots in (ii) and (iii)) to the BRXL2 centre of mass (green dot in (iv)) to define two angles. (c, d) Curve fitting of the average pixel intensities to a Gaussian model (blue line). The four parameters from the regression model are labelled as: standard deviation (SD) $\sigma$, centre $\mu$, amplitude $\alpha$ and baseline $\beta$. (f) Representative confocal images and POME measurements of polarised and depolarised markers. Magenta: pBL1::RCI2-mCherry; green: pBASL::YFP-BASL (upper left), pBRXL2::BRXL2-YFP (lower left and top right), pBASL::Myr-BRX-YFP (lower right). Open white triangles indicate the centres of the polarity domain suggested by POME. (g) Comparison of the fitted parameter values for each marker ($n = 20$ cells/marker). (h) Receiver operating characteristic (ROC) curve generated from the binary logistic regression model built using the SD, amplitude and baseline. The closer the curve is to the upper left corner, the higher the predictive power of the parameter. (i) Sensitivity and specificity curves for a range of polarisation cut-off values of SD. (j) Result of the classification of the training data set using a SD cut-off value of 60°. The region classified as polarised is marked in shaded orange. Bars, in (b, f) are 5 μm.

Applications of POME in other developmental contexts

POME was initially designed to address the challenges of measuring polarity in dispersed, asynchronously dividing, leaf epidermal cells. An automatic tool to quantify polarity, however, is useful for other developmental contexts and the simple design of POME should make it adaptable to other tissues and organisms.

In Arabidopsis, the MAPKKK YODA (AtYDA) is polarised and segregated preferentially to larger daughter cells in the stomatal lineage ACDs by BASL (Zhang et al., 2015). In Brachypodium, a YODA homologue, BdYDA1, is also required for stomatal lineage ACDs (Abrash et al., 2018). However, the Brachypodium genome does not encode a BASL homologue (Bowles et al., 2020). It was therefore unclear whether BdYDA1 would be polarised in the Brachypodium stomatal lineage. Grass stomata are arranged in cell files and exhibit a base-to-tip gradient
Fig. 2 POME reveals differences in BRXL2 and BASL polarity dynamics during asymmetrical cell divisions (ACDs). (a) Time course of BRXL2 localisation during an ACD. Cells are marked with pML1::RC12A-mCherry (magenta) and pBRXL2::BRXL2-YFP (green). Time point 00:00 (h:min) marks the formation of the cell plate. (b) Changes in SD, amplitude and baseline of the BRXL2 distribution determined by POME. (c) Plotted measurements of individual cells, each expressing either BRXL2 (top cells, 1–3) or BASL (bottom cells, A–C). Vertical dashed lines indicate cell plate formation. Solid horizontal lines indicate a SD cut-off value of 60° and all values in shaded orange are classified as polarised. The 60° SD cut-off value that achieved 100% classification sensitivity is chosen because of the lower image resolution and lower signal-to-noise ratio of time-lapse images compared with still images. For each cell, the first and last time-points showing polarisation are marked by black boxes. (d) BRX (cyan) and BASL (yellow) localisation pattern during the late G2/early mitotic phase in two meristemoids. Note that in cell 2, BRX is polarised while BASL is not. Bars in (a, d), 5 μm.
of development. Meristemoids undergo directional ACDs such that the smaller daughter cell (stomatal precursor, GMC) is oriented towards the tip and the larger one (pavement cell precursor) towards the base (Fig. 4a,b). To determine if BdYDA1 is polarised, we used POME to quantify its distribution in individual meristemoids before ACDs and in the newly formed larger daughter cells resulting from this division (Fig. 4a,b).

BdYDA1 was not polarised in meristemoids but, after ACDs, polarised BdYDA1 was detected in the larger daughter cell (Fig. 4b,c). This segregation and polarisation of BdYDA1 is consistent
with a role restricting stomatal fate and these larger daughters often wrongfully acquire stomatal identity in the loss-of-function mutant *bdyda1* (Abrash et al., 2018). The position of polar *BdYDA1*, distal to the new division plane in the larger daughter cell, is equivalent to where AtYDA localises in a BASL-dependent manner in Arabidopsis (Zhang et al., 2015). This leads to the interesting conclusion that MAPKKs can and must polarise during stomatal ACDs to ensure differential fates in different plants, but BASL may be newly recruited into this role in dicots and alternative mechanisms must exist to polarise *BdYDA1*.

The expression of *BdYDA1* in each of many contiguous stomatal cells represented a complication for automated polarity analysis. We had to rely on prior knowledge on oriented ACDs (Abrash et al., 2018) to infer in which cells and on what cell face *BdYDA1* was localised. The problem of assigning polarity to individual contiguous cells is quite common. In Arabidopsis roots, for example, cells are also arranged in files and localisation of proteins to specific faces is required for the development and physiological functions of the root. Many proteins in the root and vascular systems, especially those involved in polar auxin
transports, displays polar localization to 'shootward' and/or 'rootward' faces of the cell (Wisniewska et al., 2006; Scacchi et al., 2009; Zourelidou et al., 2009; Breda et al., 2017; Marhava et al., 2018) (Figs 4e, S4a). Determining whether a protein is present at both shootward and rootward faces in a cell within a contiguous file has been challenging.

To test whether PO-ME could be used to determine which side of the interface between two adjacent cells a protein resides, we quantified the localization of BRXL2 and the transmembrane protein PIN2 in Arabidopsis roots. BRXL2 has been reported to localize rootward in roots (Marhava et al., 2020) and PIN2 to localize shootward in root epidermis cells (Wisniewska et al., 2006). We quantified the pixel intensity at multiple concentric 'rings' of the plasma membrane. Fluorescence in the proximal membrane region (within the solid rings in Fig. 4d) is contributed by the selected (target) cell, while fluorescence in the distal membrane region (within the dashed rings) is considered to be contributed by the neighbouring cell. With this modified PO-ME protocol, we validated BRXL2’s rootward localization pattern in lateral root cap cells (Fig. 4e–g) and PIN2’s shootward localization in root epidermal cells (Fig. S4). This modified version of PO-ME can also be applied to validate bi-polar localization, when polarity proteins are present at both shootward and rootward faces. In this case, no appreciable differences will be observable between proximal and distal measurements at the membrane sections shared between the continuous cell file and the signal intensity of the polarity reporter will be higher at such membrane sections. The same principle of capturing pixel intensity of a reporter across concentric 'rings' could also be used to determine the redistribution of proteins between membrane and cytoplasm, a common response to environmental perturbation in plants (Zourelidou et al., 2009; Marhava et al., 2018).

Conclusion

Plant proteins exhibit staggering variation in cortical polar domains. They can be found linked to dispersed ACDs, in contiguous cell files aligned with organ axes, or nestled in cell corners. PO-ME can quantify dynamic polarity in these different contexts and complements other recent advances in cell segmentation and quantitative image analysis (Wolny et al., 2020). PO-ME converts a once low-throughput and qualitative procedure into a semi-automated one that is amicable to statistical analysis. It could be even more powerful when combined with other microscopy techniques such as fluorescence resonance transfer (FRET) that directly measure protein interaction.

Ultimately, we expect fluorescence microscopy and tools like PO-ME to answer some and open new questions in cell biology. In our exploration of stomatal lineage proteins in two plant species, we were able to find that BASL, while a central hub in stomatal polarity, may be preceded by BRXL2 polarity in Arabidopsis and must be supplanted by other mechanisms for establishing polar localization of the MAPKKK BdYDA1 in Brachypodium. PO-ME also allowed us to validate the rootward and shootward localization of BRXL2 and PIN2, respectively, in Arabidopsis roots. Furthermore, the utility of PO-ME is not limited to plants, as we were also able to use it to quantify PAR-2 polarity in C. elegans embryos (Hubatsch et al., 2019) (Fig. S5). The simplicity and versatility of our pipeline make it accessible to the broad research community regardless of previous programming skills.

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Author contributions
YG, RV, EW, DCB and LSC designed the project, interpreted the data and wrote the manuscript; YG, RV and EW performed the experiments; YG, RA and LSC designed the image quantification pipeline.

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Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Localisation pattern of polarity proteins used to test POME in the Arabidopsis stomatal lineage.

Fig. S2 Additional polarity parameters and classification models.

Fig. S3 Confocal images of BRXL2 and BASL localisation pattern during asymmetrical cell divisions (ACDs).

Fig. S4 Application of POME in root epidermal cells to validate shootward localisation of PIN2.

Fig. S5 Application of POME in quantifying PAR-2 polarity in the *Caenorhabditis elegans* P cell lineage during embryo development.

Methods S1 POME macro V1.

Notes S1 Brief user guide for POME.

Notes S2 POME data analysis in R.

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