Interaction Between the Dynein Anchor Mcp5 and PIP_2 in Fission Yeast

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ABSTRACT

Fission yeast show a unique phenomenon during the process of meiosis, namely the horsetail nuclear movement which occurs during Prophase 1. The force required to move the nucleus to and fro is produced by dynein which is cortically anchored via the anchor protein called Mcp5[1] which is a homologue of Num1[1] identified in budding yeast. While we know that deletion mutants of Mcp5 do not undergo oscillation[1], little is known as to how Mcp5 is distributed on the membrane and what factors affect its localisation. Budding yeast Num1 has been shown to have the highest affinity for PIP_2 in vitro[2]. Recently it has also been shown that Mcp5's mammalian ortholog NuMA, could directly partner with PtdInsP (PIP) and PtdInsP_2 (PIP_2) phosphoinositides in vitro[3].This motivated us to explore the link between Mcp5 and PIP_2 and we hypothesize that Mcp5 might depend on the localization of PIP_2 in the zygotes. Consequently, a set of experiments utilizing temperature sensitive mutants and wild-type strains in different temperature conditions were carried out. These mutant strains had abnormal levels of PIP_2 at permissive(room) temperatures which further declined on increasing temperature(restrictive environment). We have for the first time visualized the PIP2 localization in fission yeast and have characterized this profile with regard to Mcp5 localization at permissive temperature. Future work will be focused on elucidating the relationship between depletion of PIP2 and Mcp5 localization and its effect on the process of nuclear oscillations. Lessons learned from fission yeast can potentially help in characterizing similar components in higher organisms in order to understand complex cell-division.

Keywords:
Permissive; Restrictive; PIP_2; Mcp5; Dynein.

1. INTRODUCTION

There are two mating types present in fission yeast : h+ and h− , which are alleles of the mat1 locus. Normal cells are haploid and do not undergo meiosis/mating unless they are starved for nitrogen. After conjugation of the two opposite mating types, a banana-shaped zygotes is formed which immediately enters meiosis and sporulates to produce four spores (called the zygotic ascus). There is another class of cells which can change between h+ and h− mating types and these are known as h90. They are called so because 90% of cells can typically switch their mating type. Also, in a culture of h90 cells, about half of the cells will behave as h+ and half h− because of this switching [4].

In a typical zygote, the two nuclei of the parental cells fuse into one and the fused nucleus starts to oscillate from one end of the cell to the other [5]. These oscillations have a period of about 10 min, last for several hours and immediately lead to sporulation [5]. The oscillations are important for proper chromosome recombination, pairing and spore capability [6, 7]. Some of this has even been demonstrated as in the case of chromosome pairing and

[1] Num and Num1p refer to the same protein

recombination [8]. The movement depends on the cortically anchored minus end-directed motor protein dynein [6] and also on microtubules [9].

By responding to load forces, the dynein motors dynamically reorganize in the cell and attach to the microtubules coming out of the front of the moving SPB, thus helping in generating oscillations [10]. Dynein diffuses along the microtubule initially but only upon finding and consequently binding to the cortical anchor it gets activated and switches to its usual minus end directed movement[11]. Cortical dynein, generates a pulling force using an anchor protein called Mcp5 which is localized at the cell cortex, probably via attachment through its PH domain. Therefore, Mcp5 helps in the cortical sliding of microtubules via anchoring the dynein–dynactin complex. [1]

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a phospholipid of the plasma membranes and plays a key role in various processes. [12] The products of the PLC catalyzation of PIP_2 are inositol 1,4,5-trisphosphate(InsP_3; IP3) and diacylglycerol (DAG),which function as secondary messengers.
Visualization of PtdIns(4,5)P_2[PIP_2] is achieved with the help of the pleckstrin homology (PH) domain of PLCδ1 fused to GFP.[13] Using only the PH domain doesn't allow the above catalyzation reaction to proceed but at the same time PIP_2 is successfully bound to the GFP.

Phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks) are a group of kinases that catalyse the production of PtdIns(4,5)P2[14]. Fission yeast contains a gene its3$^+$ which encodes a functional homologue of Phosphatidylinositol 4-phosphate 5-kinases PI(4)P5K called Its3.The Its3 mutant strain used in the set of experiments(KP167) was operated in two sets of temperature conditions, namely, permissive (25-28) and restrictive(33-36){In Degree Celsius}. Even at permissive temperature, Its3 mutant cells contained about 10% of the amount of PI(4,5)P2 found in wild-type cells, indicating that a mutation has caused a significant decrease in PI(4)P5K activity of Its3. A shift to the higher temperature (restrictive) induced a further fall in the level of PI(4,5)P2 suggesting that activity of mutant Its3 PI(4)P5K decreases even further upon changing the temperature. However, Its3 mutant cells grow almost normally at the permissive temperature even with reduced levels of PIP2, but stop growing a few hours after shift to restrictive temperature. [15]

The Num1p PH domain of S.cerevisiae, showed a good PtdIns(4,5)P_2 binding specificity based on some in vitro binding assays [2]. In vivo studies in yeast mutants that permitted targeted manipulations of phosphoinositides concluded that Plasma membrane targeting of Num1p was dependent on PtdIns(4,5)P_2. Also Num1 showed very little movement from the membrane to the cytoplasm after PLC activation [13]. Since Mcp5 is a homologue of Num1, it is possible that it too exhibits this behavior shown by Num1 and possibly even more so.

We hypothesize that Mcp5 binds to PIP_2 and hence anchors onto the membrane and the above literature gave us the motivation to work to find the interaction between Mcp5 and PIP_2. We analyzed the PIP_2 and Mcp5 fluorescence intensity at the perimeter of the zygotes and studied the distribution patterns that follow. By combining experiments and image analysis, we have characterized the interaction between Mcp5 and PIP2 at the permissive temperature (for all strains) and restrictive temperature (for wild-type strains).

2. MATERIALS AND METHODS

The strains used in the study are given in table 1. Standard methods of fission yeast culturing and mating were followed [4].

2.1. Cell Culture and Meiosis Induction

Cells were grown on Yeast Extract [4] with appropriate supplements.

Inducing Meiosis- In case h^+ and h^- mating types, suspend half-toothpick of h^+ and half-toothpick of h^- in 100 µl of 0.85% sodium chloride and spotting onto Malt Extract Agar (MEA) plates [4]. In case of h90, the procedure is the same, except one just suspends a toothpickful of only h90 cells. The plates are then either put inside the incubator(to check growth at restrictive temperature) or inside the hood(to check growth at permissive temperature) for a 7-10 hours for h90 cross and 12-18 hrs for h+ crossed with h-.

2.2. Preparation for Imaging

First, we checked for the formation of zygotes under a compound microscope. We took 100ul of EMM-N[2] and suspended some of the crossed strain in it and saw it under a microscope. Only if there was a sufficient density of zygotes did we proceed forward.

For imaging, cells were suspended in distilled water and spun down twice. After removing water, the cells are re-dissolved in Hoechst stain for 20 minutes.
Meanwhile a glass bottom cover dish was prepared and had lectin coated on it as only then would the yeast cells stick onto it.. After 20 minutes of Hoechst staining is over, we spun down and then re-dissolved the cells in EMM-N. The cells are then transferred to the lectin-coated glass bottom cover dish and allowed to stick to the glass for 5 minutes The unbound cells are then washed out with EMM-N and finally imaging is performed either at room temperature (to check behavior in permissive conditions) or at a higher temperature(to check behavior in restrictive conditions)[3].

Imaging was done in The InCell Analyzer 6000, which is a high-content, laser line scanning confocal microscope. The objective lens used was Nikon 60X/0.7, Plan Fluor, ELWD, Core Collar 0.5-1.5,CFI/60. For viewing the Hoechst stain, the channel settings used were UV(Excitation-405nm) and DAPI(Emission) with an exposure of 0.1s, image mode being 3-D(Slices:15,Step size:0.5) with the laser power being 100%. Similarly, for visualizing GFP, Blue (Excitation-488nm) and FITC (Emission) channel settings were used with an exposure of 0.6s, image mode being 3-D(Slices:15,Step size:0.5) with the laser power being 100%. When imaging was done at restrictive temperature, the plate and lid heater of the InCell Analyzer was set to 33(degree Celsius) at least an hour prior to imaging and the cover dish was kept inside the lid throughout while imaging.

2.3. Analysis of Images and Data

Images were analysed in ImageJ and the final data analysis was done in Matlab. As mentioned in section 1, the intensity of PIP_2 and Mcp5 was measured along the perimeter of the zygotes. A model was constructed by us as shown in Figure 1 which would then be helpful in studying the pattern distribution .Figure 1 depicts a typical banana shaped zygotic ascus. As depicted, the part of the nucleus which is long and pointed is referred to as the leading edge. We have henceforth identified 4 different points on the perimeter of the zygote which fall into two types. The first set refers to the “pole points” (located farthest from the center of the zygote) and the other set consists of the “center points” which lie near the center portion of the zygote, depicted by P1, P2 and C1, C2 respectively. P1 refers to the point towards which the nucleus was moving, i.e. the pole towards which the leading edge points. Now going in any direction (anti-clockwise or clockwise), we name the points as C1, P2 and C2. This naming scheme is important in the final data analysis.
Images were accordingly stacked, projected (both with Maximum Intensity and Sum of Slices projection types) and

[2] Edinburgh minimal medium-Nitrogen

[3] If not mentioned specifically, restrictive temperature used in the experiments was 33(degree Celsius)

studied using ImageJ. Only zygotes were identified and isolated as region of interests (ROIs) and fluorescence intensity profiles were plotted for all the zygotes. Matlab was then used to analyze these intensity values as a function of distance along the perimeter of the zygote. All the zygotes were normalised for their length (which here refers to distance and can be thought of as distance from P1 all the way back to P1 itself) and individual maximum intensity values. This was done using the assumption that all the zygotes would have more or less the same banana like shape. The normalisation for intensity was carried out as some zygotes could have produced more amounts of PIP_2 or Mcp5 as compared to others due to some reason. After this the mean of the normalised intensity values of all zygotes (for the same normalised distance) was calculated.

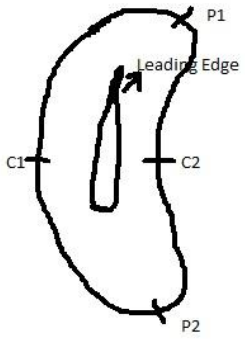


Figure 1.

Finally, all these normalised mean intensity values (of PIP_2 or Mcp5) were plotted against normalised distance. Note again, in this context we measure distance as 0 when we are at point P1 which then progressively increases as we move around the zygote we are studying.

Name	Genotype	Mating Type
KP692(Its-3 mutant)	leu1 ura1 its3-1	h^-
KP167(Its-3 mutant)	leu1 its3-1	h^-
AJC-D40(PH-GFP tagged)	leu1-padh1-PHP-GFP-leu1-32	h^+
FY16855(Mcp5-GFP tagged)	ade6-M2106 mcp5::[mcp5-GFP-3'UTR-Lys3+]	h^-
FY16854(Mcp5-GFP tagged)	his2 leu1-32 ura4-D18 mcp5::[mcp5-GFP-3'UTR-Lys3+]	h^+
L972(Untagged Wild Type)	-	h^-

Table 1. Yeast strains used in experiments (with description)

3. RESULTS AND DISCUSSION

3.1. Results

It was observed that in permissive temperature conditions, the zygote formation takes place in roughly 18 hours whereas in restrictive temperature the time taken was 12 hours for all the strains of mating types h^+ and h^- (neglecting some variation). This suggested to us that mating took place faster at higher temperature conditions. The Its3 strains showed similar trends in permissive conditions. However in restrictive environment zygote formation was either completely hindered (KP167) or partially affected (KP692). Recalling from section 1, in case of the Its-3 strains possibly the only thing changing from the permissive to the restrictive environment is the amount of PIP_2 only. Therefore it might be the case that there is not enough PIP_2, possibly for Mcp5 anchoring. Hence, for this reason, there might exist a critical concentration of PIP_2 above which zygote formation is possible.

In the following sub-subsections, a summary of the all the analyzed data has been provided. The focus was to find a unique pattern of Mcp5 and PIP_2 distribution in the zygotes and draw conclusions thereafter. The fluorophore used was GFP and it was tagged to both PIP_2 and Mcp5.

The following plots list out data from various crosses done. Note that in the plots listed here, we have followed the exact same procedure as we had discussed back in section **2.3**.The procedure first included normalising each zygote for its length which was done by dividing the distance of each point by the perimeter of the whole zygote. Finally we get what appears as normalised perimeter in the plots. Next each zygote was normalised for the fluorescence intensity observed along the perimeter. This was done again by dividing intensity of each point along the zygote by the maximum intensity observed along the perimeter. Following this step, the mean of the normalised intensity values of all zygotes was calculated. Finally, all these normalised mean intensity values were plotted against normalised distance calculated earlier using data binning techniques.

One important thing to note is that P1 is 0 and 1.01, C1 is 0.25, C2 is 0.75 and P2 is 0.5(roughly) on the x axis which here, in these plots, stands for normalised perimeter. Thus we get a plot of how fluorescence intensity is varying across the zygote as a function of distance travelled around it.

Note- In the sub-subheadings that follow, the letter n symbolizes the number of zygotes.

3.1.1 AJC-D40 X[4] L972(permissive)[n=31]

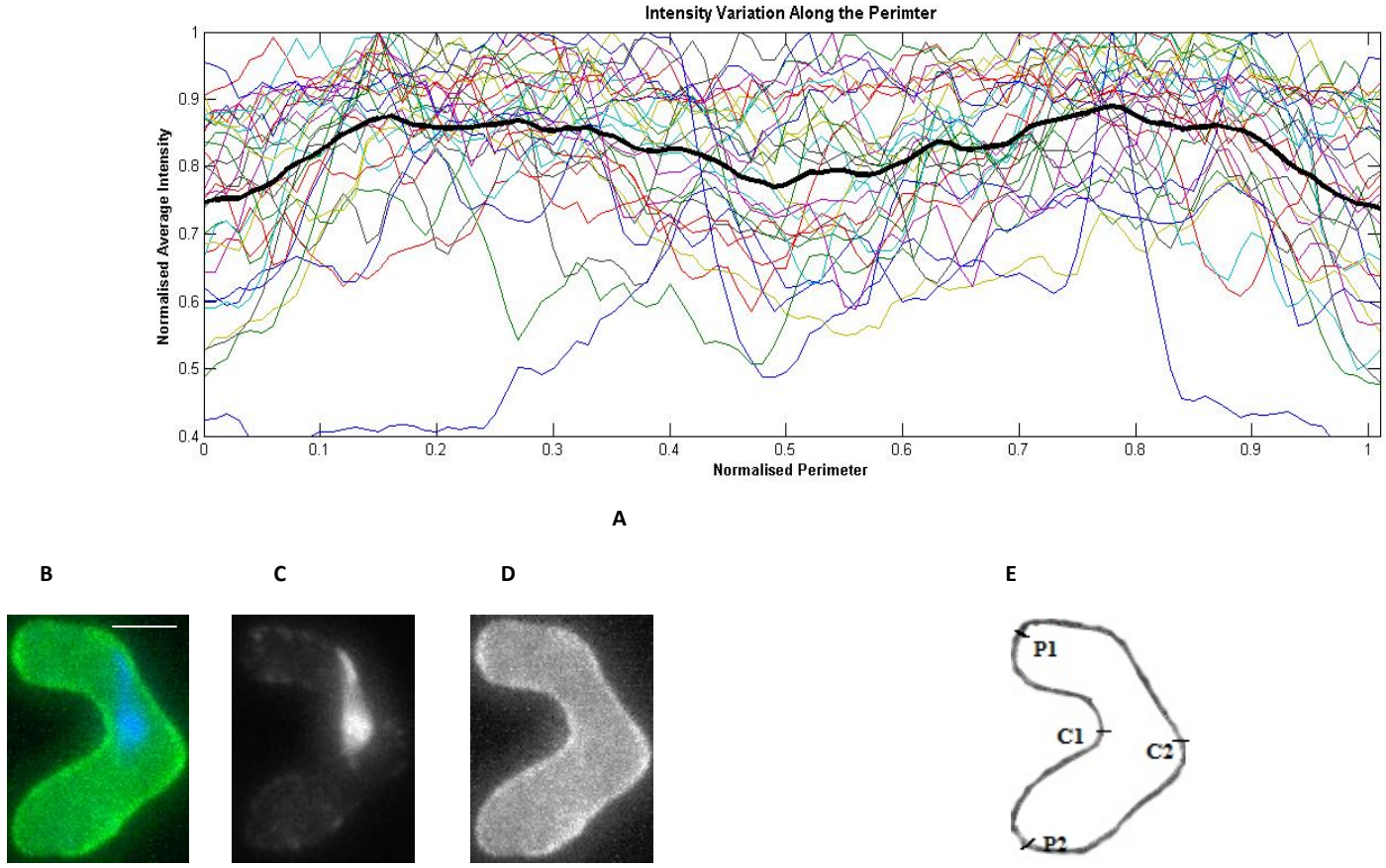


Figure 1.

The scale bar (a white line shown in the composite image) equals 2µm.

(A)Here the bold line is the final curve consisting of the normalised mean data points(for all zygotes). The thinner lines represent normalised data points for each zygote.
(B)Composite Image of (C) and (D). Nucleus is depicted in blue while PIP_2 is depicted in green.
(C)Nucleus(Hoechst stained)
(D)PIP_2(GFP tagged)
(E)Zygote outline with all points labelled

[4] The X symbol stands for a cross between 2 strains on either side of the symbol

3.1.2. AJC-D40 X L972(restrictive)[n=37]

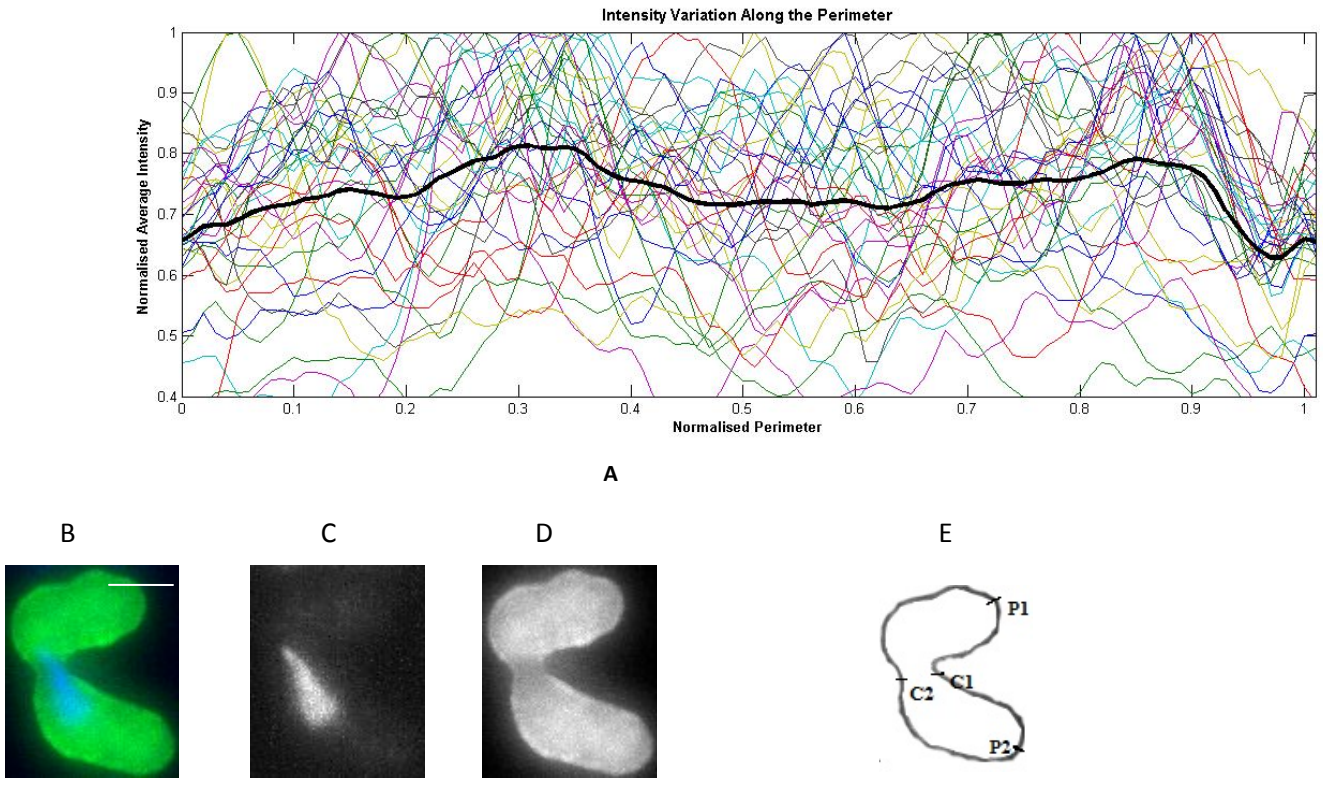


Figure 2.

The scale bar (a white line shown in the composite image) equals 2µm.

(A)Here the bold line is the final curve consisting of the normalised mean data points (for all zygotes). The thinner lines represent normalised data points for each zygote.
(B)Composite Image of (C) and (D). Nucleus is depicted in blue while PIP_2 is depicted in green.
(C)Nucleus(Hoechst stained)
(D)PIP_2(GFP tagged)
(E)Zygote outline with all points labelled

3.1.3 FY16854 X FYI6855 (permissive)[n=30]

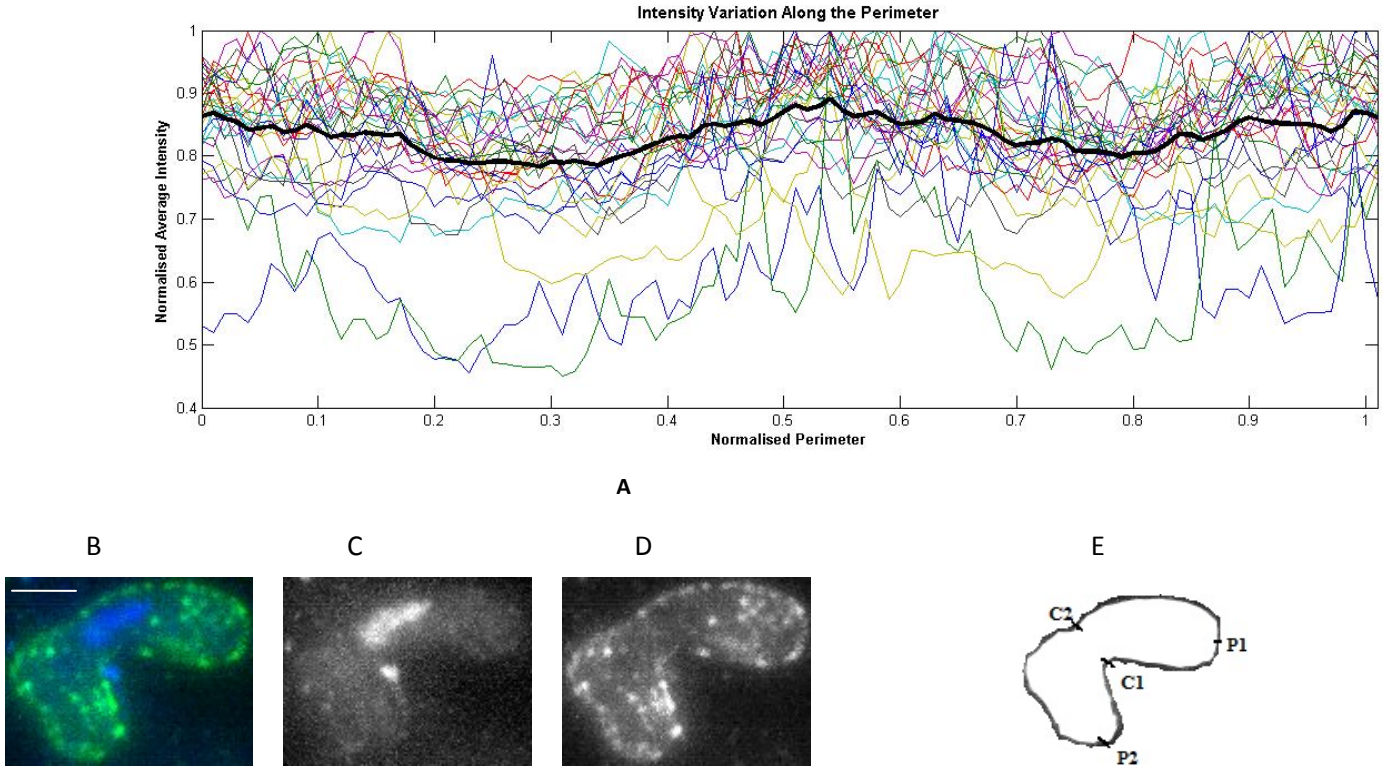


Figure 3.

The scale bar (a white line shown in the composite image) equals 2µm.

(A)Here the bold line is the final curve consisting of the normalised mean data points(for all zygotes). The thinner lines represent normalised data points for each zygote.
(B)Composite Image of (C) and (D). Nucleus is depicted in blue while Mcp5 is depicted in green.
(C)Nucleus(Hoechst stained)
(D)Mcp5(GFP tagged)
(E)Zygote outline with all points labelled

3.1.4. FY16854 X FYI6855 (restrictive)[n=26]

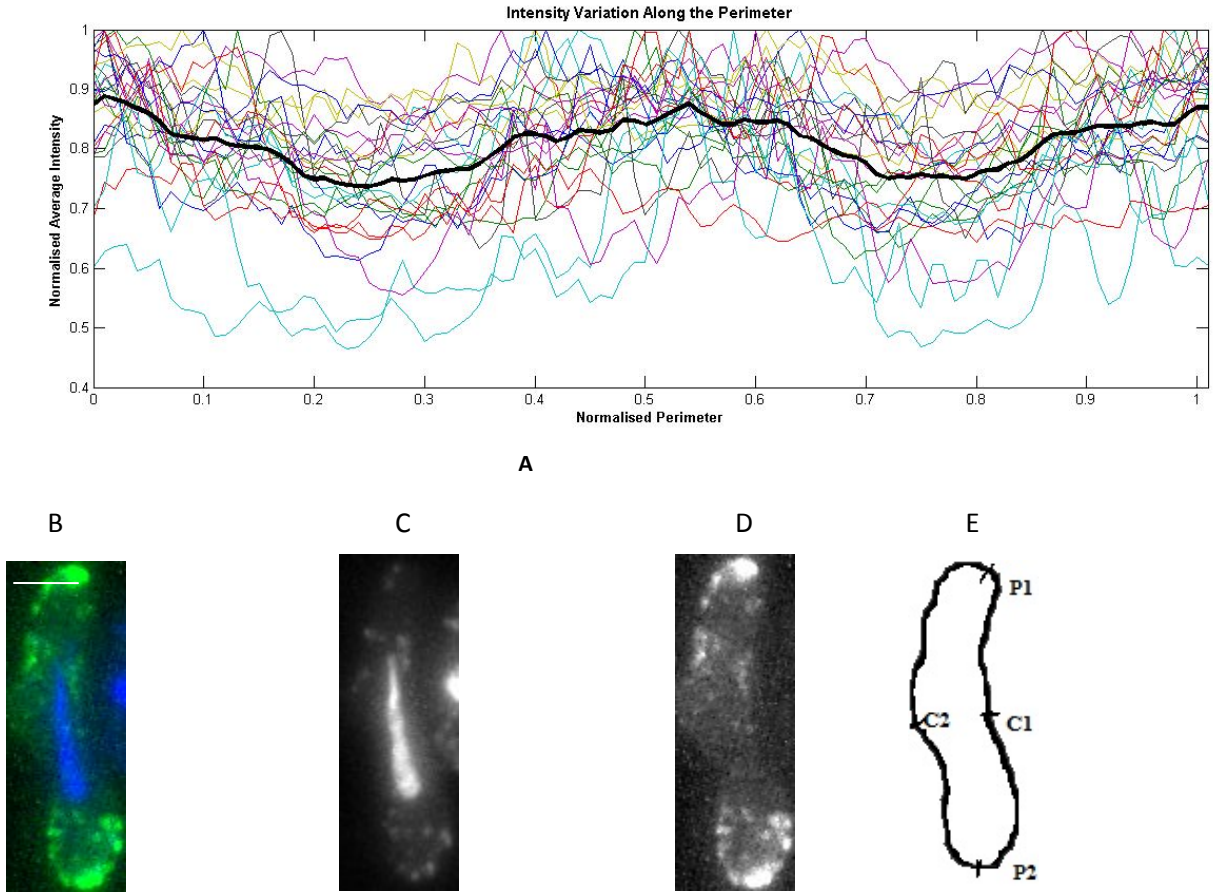
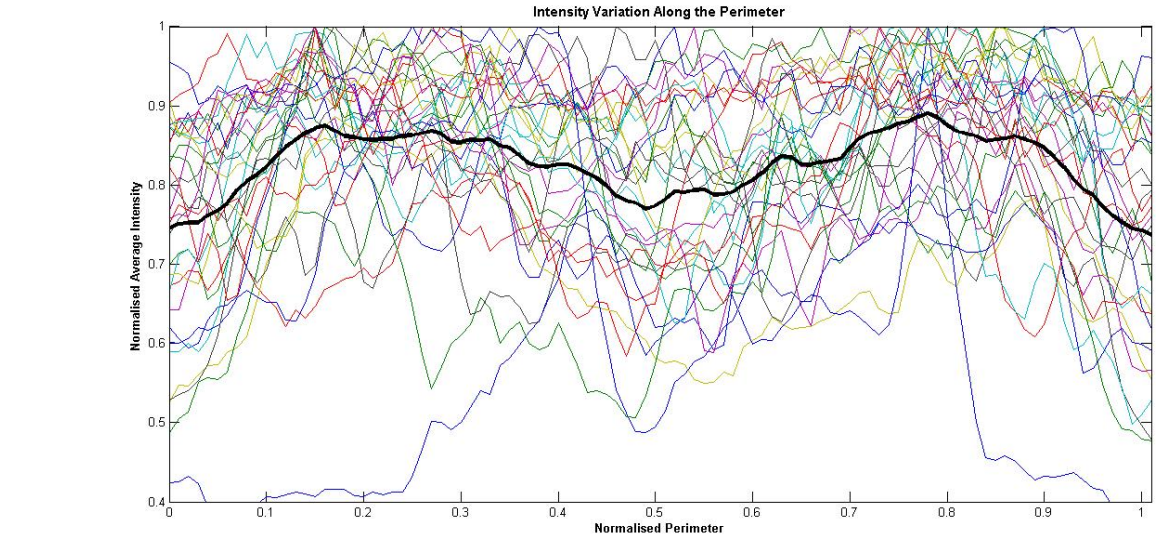


Figure 4.

The scale bar(a white line shown in the composite image) equals 2µm.

(A) Here the bold line is the final curve consisting of the normalised mean data points(for all zygotes). The thinner lines represent normalised data points for each zygote.
(B)Composite Image of (C) and (D). Nucleus is depicted in blue while Mcp5 is depicted in green.
(C)Nucleus(Hoechst stained)
(D)Mcp5(GFP tagged)
(E)Zygote outline with all points labelled

3.1.5. AJC-D40 X KP167 (permissive)[n=35]



A

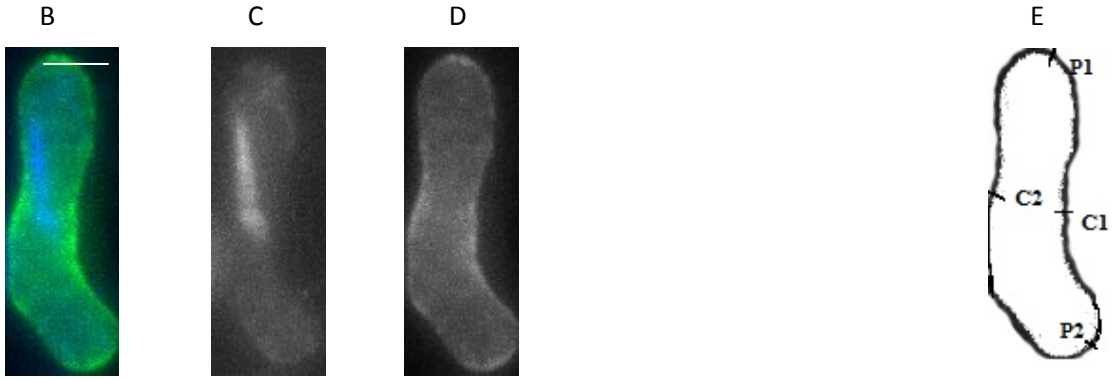


Figure 5.

The scale bar(a white line shown in the composite image) equals 2µm.

(A)Here the bold line is the final curve consisting of the normalised mean data points (for all zygotes). The thinner lines represent normalised data points for each zygote.
(B)Composite Image of (C) and (D). Nucleus is depicted in blue while PIP_2 is depicted in green.
(C)Nucleus(Hoechst stained)
(D)PIP_2(GFP tagged)
(E)Zygote outline with all points labelled

3.1.6. AJC-D40 X KP167 (permissive+restrictive)[n=4]

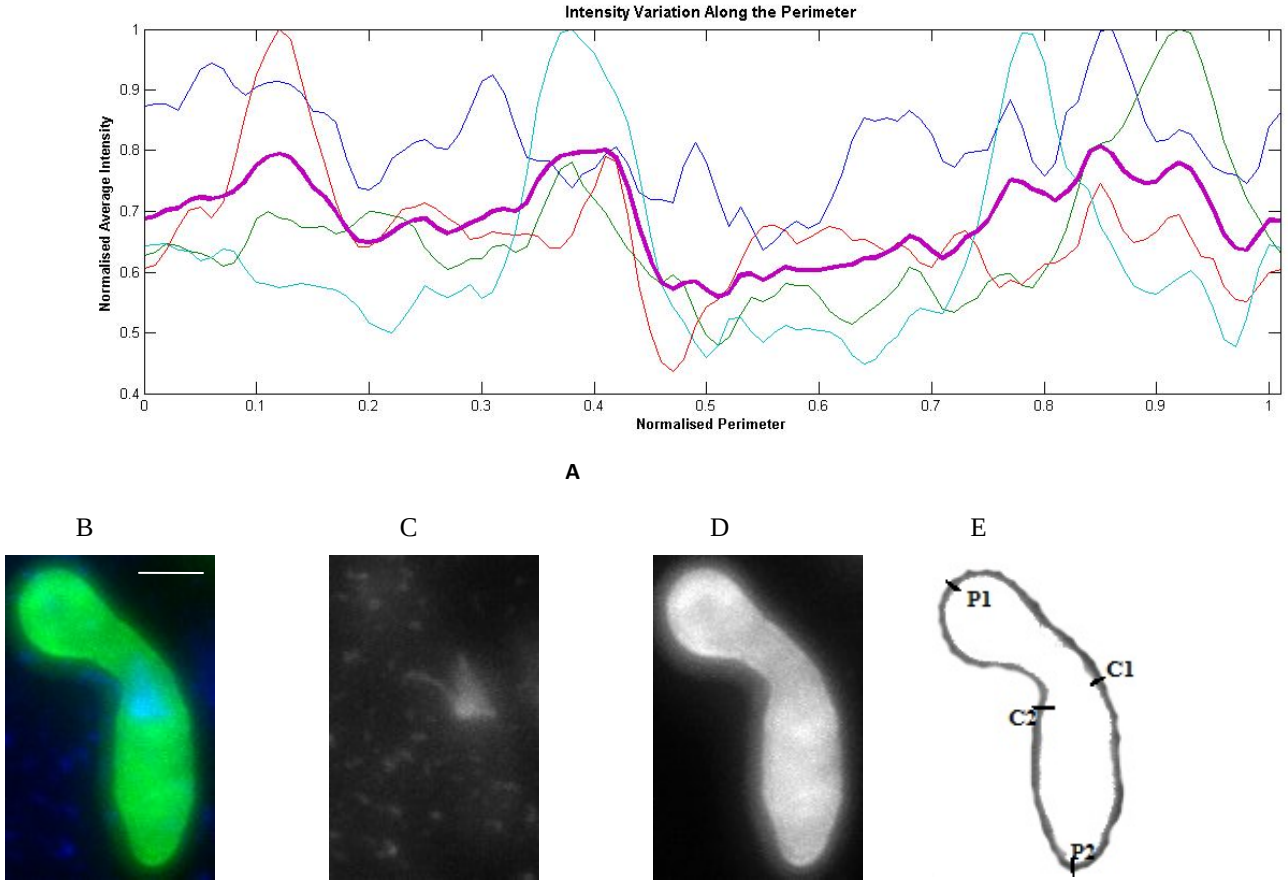
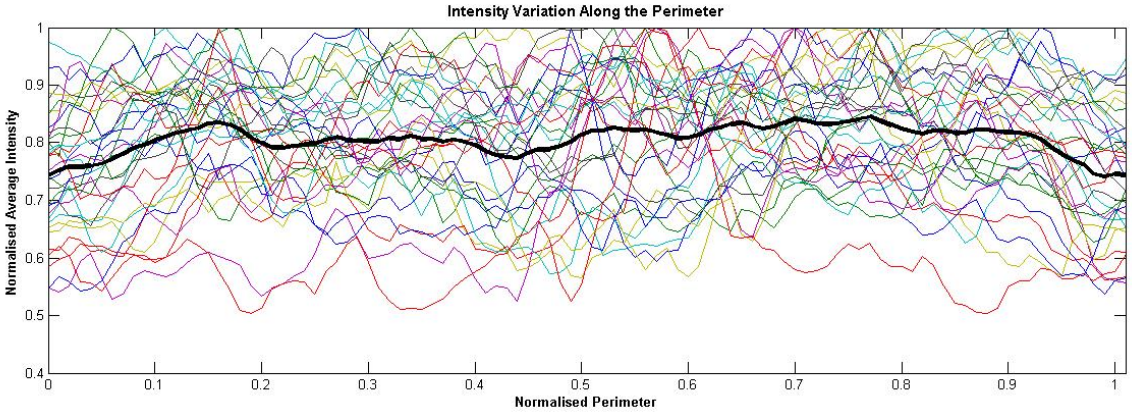


Figure 6.

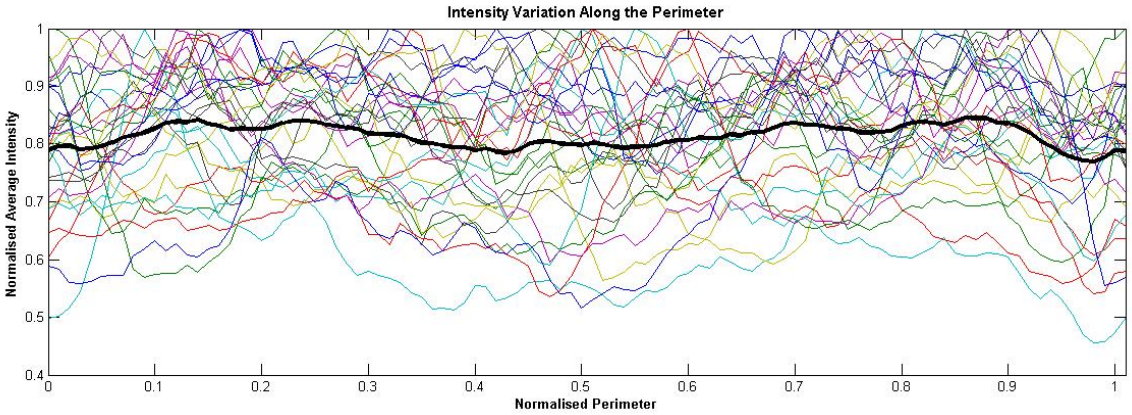
The scale bar(a white line shown in the composite image) equals 2µm.

(A)Here the bold line is the final curve consisting of the normalised mean data points(for all zygotes). The thinner lines represent normalised data points for each zygote.
(B)Composite Image of (C) and (D). Nucleus is depicted in blue while PIP_2 is depicted in green.
(C)Nucleus(Hoechst stained)
(D)PIP_2(GFP tagged)
(E)Zygote outline with all points labelled

3.1.7. AJC-D40 X KP692 (permissive)[n=32(A)+30(B) respectively]



A



B

C D E F

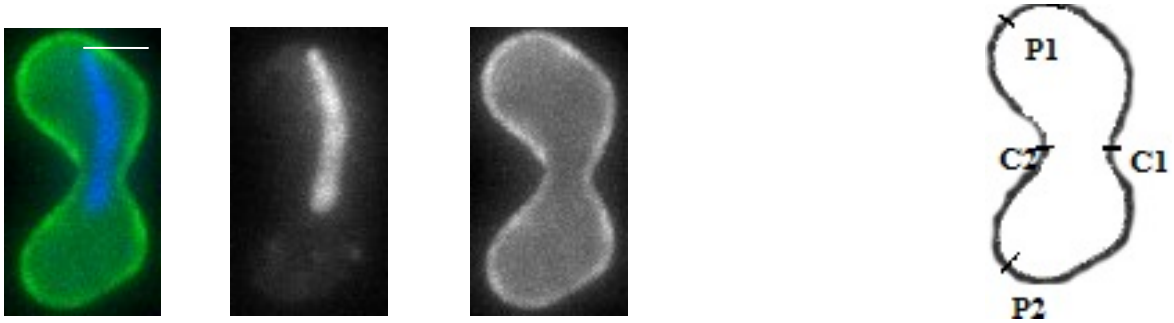


Figure 7.
The scale bar(a white line shown in the composite image) equals 2µm.

(A) Here the bold line is the final curve consisting of the normalised mean data points. The thinner lines represent normalised data points for each zygote.
(B) Here the bold line is the final curve consisting of the normalised mean data points. The thinner lines represent normalised data points for each zygote.
(C) Image of (D) and (E). Nucleus is depicted in blue while PIP_2 is depicted in green.
(D) Nucleus(Hoechst stained)

(E) PIP_2(GFP tagged)
(F) Zygote outline with all points labelled

3.2. Discussion

In this section the data from the plots will be analysed and discussed.

In figure 1, from the intensity profiles, we can clearly see that PIP_2 concentration was localised much more at the center and starting depleting as we moved towards the poles. This sort of distribution is not found in a normal cell where it is more or less uniformly distributed along the perimeter of the cell.

In figure 2, we can see that at restrictive temperature, there does seem to be a bit of a change in the relative peak heights, but the trend remains more or less the same as what we saw from figure 1. Here the concentration at the centre seems to have increased relatively more than in the case of permissive environment and hence the plot seems more uniform but we are not very clear as to why.

In figure 3, what we observe is that the Mcp5 distribution was exactly opposite to what we found in case of PIP_2. It was present mostly around the poles but becomes lesser at the center.

In figure 4, what we can see is that again at the restrictive temperature, we see a similar kind of behaviour as seen in permissive temperature. Here the relative peak heights also seem to be the same as compared to the permissive case. In anycase from figures 1,2,3,4 it is clear that the distribution of PIP_2 and Mcp5 respectively, effectively remains the same in case of wild-type cells.

In figure 5, we can see that in the permissive conditions, KP167 gives a more or less similar distribution of PIP_2 as compared to the distribution in figure 1. Therefore, even though the PIP_2 content is quantity-wise less in these strains , the distribution profile remains the same as what was found in case of wild type cells.

In figure 6, as one can see, the final curve is not very smooth. One obvious reason is that the number of zygotes that could be found out was very low. This may be explained by the conditions in which these crossed strains were grown. The cells were initially crossed in permissive temperature and allowed to stay for a while. Then the cells were shifted to restrictive environment just before the zygote formation could start and stayed there till the end of their standardized “zygote producing” time. As noticed earlier, these conditions inhibit zygote formation in the Its-3 strains (here KP167). Thus, there was a reduction in the number of zygotes seen as compared to if it was only in permissive conditions. One thing one will notice upon seeing the plot is that we again see a rise in intensity around the center which then reduces at the poles, again something consistent with the profiles for PIP_2 we had got earlier(Figures 1,2,5). Apart from playing a crucial role in zygote formation, this suggests that PIP_2 has a characteristic profile in all zygotes, perhaps to facilitate a proper positioning of Mcp5 along the cortex which is common for all types of strains.

In figure 7, we can see that the other mutant strain, KP692, gave a very different plot when analysed for the first time (Fig 7A).The intensity profile did not show much variation throughout the perimeter, a feature not observed in the earlier plots for PIP_2. Hence a second analysis was carried out to ensure consistency of results (Fig 7B). The second one too came out almost the same as the first. However, if we analyse more closely we will observe that both the plots show the same kind of trend that we have observed in PIP_2 levels for KP167 and L972, i.e. there is more at the center than at the poles. Although we notice that the relative peak to peak distance in the plot is very less, i.e. the minimum and maximum intensity values in the plot are not extremely different (when we compare it to the other strains in permissive conditions).

Apart from the points already raised, one other major point which can be taken away from the data is that there seems to be a competition between the Mcp5 PH Domain and the PLCδ1 PH domain for binding to PIP_2. This is because from the plots, we clearly see that at the centre points, there a lot of PLCδ1 PH domain(fused to GFP) bound to PIP_2 whereas Mcp5 seems to present more towards the pole areas. It is very much possible that Mcp5, which should have much more affinity for PIP_2 than the PLCδ1 PH domain, displaces the PLCδ1 PH domain from the areas near the poles (where it localizes) in order to bind to PIP_2. We thus lose the GFP signal near the poles and end up arriving at a distribution pattern seen in the figures. This seems reasonable as from the images (See Figure 4) we see a lot of Mcp5 near the poles. This increased concentration of Mcp5 therefore now interacts with more and more PIP_2 and as a consequence displaces the PLCδ1 PH domain. In areas where there is less of Mcp5, i.e. near the

center, we find that the GFP intensity is more. This again must be because less of Mcp5 interacts with less of PIP_2 and as a consequence displaces less of the PLCδ1 PH domain already bound to PIP_2.
Notice that the PLCδ1 PH domain is already bound to PIP_2 as the strain had been genetically modified. So Mcp5, which is only produced inside the fission yeast during the nuclear oscillation stage, will have to displace this PH domain before it can bind to PIP_2 itself.
We thus observed and characterised Mcp5 and PIP_2 distribution in zygotes which may explain how Mcp5 interacts with PIP_2.

4. CONCLUSION

In conclusion, after studying the distribution patterns of PIP_2 and Mcp5 we have characterized their interaction. This was done using the intensity patterns of the two (PIP_2 and Mcp5) obtained from the zygotes. Zygotes have a characteristic intensity profile(along the perimeter)associated with both Mcp5 and PIP_2 and both seem to depend on each other. Although some more proof is still required to establish this connection once and for all, but from results obtained from our work it does seem that the hypothesis should work out correctly.
There is a lot of future scope in this work. Further thought can be given to h90 strains and PIP_2, Mcp5 distribution can be studied in them. Also, one can then deplete PIP_2 and study the effect on Mcp5 localisation. More work can be done to study some other key players in the process of oscillation and similar intensity based analysis can be done in them to study their distribution patterns. The data and image analysis techniques can be used to study fluorescence intensity variation along a boundary (most probably a membrane) in other types of systems.

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