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Mating yeast cells use an intrinsic polarity site to assemble a pheromone-gradient tracking machine

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March 15, 2019

Re: JCB manuscript #201901155

Dr. David E Stone
University of Illinois at Chicago
Biological Sciences
Molecular Biology Research Building
900 South Ashland
Chicago, Illinois 60607

Dear Dr. Stone,

Thank you for submitting your manuscript entitled "Mating yeast cells use an intrinsic polarity site to assemble a pheromone-gradient tracking machine". We sincerely apologize for the delay in communicating our decision to you. We were waiting for input from a third referee who committed to the review but ended up not being able to send us any comments, and this significantly delayed the process. Thank you very much for your patience with the review process. The manuscript has been evaluated by two other expert reviewers, whose reports are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers both found that the work contains interesting and novel observations. However, both also raised a number of important reservations and issues with the degree to which the main conclusions are supported by the data. These are significant issues from experts in the field that we editorially find valid and important.

To provide more detail, Rev#1 and #2 did not feel that the claims are justified based on the imaging provided and requested a number of additional controls and more convincing data (Rev#1 Points #1, #2, #3; Rev#2 point #2, #4, #5, #6). Rev#1 was concerned that the timing analyses of protein detection may not represent the actual ordered arrival of these molecules at the polarity site (#5). The ref was critical of the quantifications and how accurate they reflect the data (second major section in major comments and also 'Figure 5' paragraph) due to concerns over the averaging methods. Rev#1 felt that additional analyses are needed to validate the fig 4 conclusions ('Fig 4' paragraph). Rev#2 wondered about the role of protease Bar1 in gradient sensing/tracking in the context of the model (#1) and recommended work to make the computational analyses more accessible and complete (point #7). A substantial amount of work would be needed to tackle these points in depth and rigorously and provide strong and definitive conclusions.

We are interested in this topic of research and appreciated the referees' interest as well. Should the work reach the level of resolution, strength, and mechanistic definition requested by the reviewers in their remarks below, we would be open to considering it at the journal for re-review by these experts. We would note that Rev#2 point #1 is interesting but is less of a priority in revision, in our view. Our recommendation is to focus the revisions on bolstering the current dataset and model, especially the assumptions and robustness (whether small changes in certain parameters would invalidate the simulation), as was pointed out by Reviewer #1. This reviewer additionally had problems with the quality of some of the data and interpretations, and all of these points together

with Reviewer #2's particular issues with the data need to be addressed thoroughly for reconsideration at the journal.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Ian Macara, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this paper Wang and co-authors combine imaging data and a mathematical model to describe the assembly, re-positioning and stabilization of pheromone-signaling machinery at polarity sites during the sexual lifecycle of budding yeast cells. The imaging is in line with recent data that cells sense pheromone gradients at a polarized site and there are interesting novel observations, notably the double fusion of rsr1Δ cells with two partners. However, it lacks rigor in quantification, which lower the confidence in the observations. The model proposes differential sensing by receptors within a single polarize patch, but it is extremely complex and the assumptions and robustness are not explained.

In my opinion, the work needs substantial revision before it can be considered for publication.

Major comments:

The imaging data clearly shows localization of the pheromone receptor and associated proteins (Gbeta, Far1 and Sst2) at a mobile patch. The authors however extract considerable additional information, which I think is poorly supported at this time:
1. Site of initial polarization: this should be co-labelled with a stable transmembrane marker of the default site to claim that it is the default site. In several examples, what dictates the choice of the position and the timing is unclear. For instance, in Fig 1 in the daughter cell of the Gbeta timelapse (not marked), the signal seems to be at very similar position at both 0' (marked as cytokinesis) and 5' (marked as polarity establishment).
2. More generally, it is unclear how the authors define the position at which to put the arrowheads. Is it the center of distribution or the brightest pixel? For instance, in the Ste2-GFP example of Fig 1 at timepoint 55', the arrowhead seems very decentered. This is important to explain how the start of tracking is defined.
3. Timing since cytokinesis: a cytokinesis marker should be used for co-localization. It is currently unclear how the timing of cytokinesis is precisely defined.
4. "Pause" of the patch at polarity establishment: In the few figures that provide images of this early timepoints, the distribution of Far1 and other components do not look completely static. It could well be that patch movements are already happening there (perhaps less directed).
5. Sequential arrival of proteins at the polarity site: Fig 3 is difficult to evaluate without seeing the imaging data. First, without two-color imaging, it is difficult to relate differential timings. In addition, I fear that this presented order of events is greatly influenced by the signal to noise levels of the various fluorescent reporters studied. For instance, the Sst2 cytosolic pool shown in Fig 1 appears very large, which likely precludes identification of small cortical signals, which may appear sooner than is reported in this analysis. Similarly, if receptor localization depends on new expression, the folding rate of GFP will influence the timing of detection. Thus, I am not sure that these differential timings of detection represent a real ordered arrival at the polarity site.
6. Rate of tracking: the text states tracking occurs at a constant rate, but no data is shown to support this claim.

The quantifications performed (Fig 2 and others) do not seem to accurately report the actual data. The images do not represent such peaked distributions. I suspect that the averaging method used skews the data. This is likely the result of centering distributions on the max, rather than on an independent marker of default and chemotropic sites. Using co-imaged independent markers to

align the data would be necessary. In addition, providing kymographs of the cell membrane for several individual cells could help interpretation of the data. It is also unclear what the error bars report. Please make sure you report SD, not SEM, which carries no information regarding biological variation and is un-interpretable without knowing the number of samples.

(Fig 4) The authors then use far1-H7 and ste2-7xR/6SA mutants, concluding that the localization of Gbeta is distinct in these two mutants. This is confusing: both mutants are reported not to orient growth up the pheromone gradient. Far1-H7 cells are reported to use the default site. The authors claim here that the Gbeta does not localize at all to the default site. This is difficult to evaluate without seeing the shmooing and fusion events. The image shows spreading of the Gbeta signal, which does not look very different from what is shown in case of the ste2-7xR/6SA mutant in the same figure, in which Gbeta is described to relocalize to the default site. This also strengthens the need for an independent marker of the default site. I would like to see more examples, quantification, and imaging until cell fusion. Moreover, in ste2-7xR/6SA cells, is it really still the default site by the time the cell shmoos or did the signal translocate, as it seems?

(Fig 5) It looks clear that the endocytosis distribution is broader than that of secretion, similar to what has previously been shown during budding. However, Sla1 fluorescence signal is weak and I am not convinced by the very specific statement that endocytosis is at the back of the distribution or surrounding the chemotropic site at fusion time. Plotting myself some of the distributions shown in the images does not immediately recapitulate the distributions shown in the example graphs. Maybe there are normalization issues to the quantification.

Model: The model is very complex with very many parameters, with unclear assumptions. There are also issues in bulking Gbeta localization with the site of secretion, which is well known to depend on Cdc42 activity. For instance, McClure et al had shown that the Gbeta co-wanders behind the Cdc42 polarity patch when uncoupled from it rather than with it, showing that these are two distinct entities. The well-characterized positive feedback regulations of Cdc42 are not taken into account. I do not think these can be ignored. It is also not clear how receptor dimerization (which is introduced out of nowhere) influences the model. To be understandable and useful, the following needs to be made explicit:
1. What are the assumptions of the model?
2. What are the parameters? Table S7 lists variables and parameters, but at least the right-hand side of the table is not obviously understandable. Since I do not know what all the parameters are (krs, krl, krlm,...), I cannot evaluate the assumptions behind the chosen values.
3. Which parameters are constrained by experiments? Which ones are free?
4. How robust is the model? I am concerned that the previous version of the model was sensitive to a 10% change in Gbeta:receptor ratio. Here, the authors use a 6:5 ratio, which they claim is based on experiments. However, fluorescence measurements can estimate an order of magnitude, but not a precision of 10-20%, especially with weak signals as here. If the model hinges on this specific ratio, this should be taken with great caution.

(Fig 7) The double mating phenotype of rsr1Δ cells is very interesting and this example clearly shows double polarization of Ste2. In the other examples, representing 99% of the cells, Ste2 localization is not convincing me of the presence of multiple discrete polarity patches. It does not look very different from the WT situation described in Fig 1. Use of a distinct marker of polarization (Bem1 for instance) may be more convincing. The main difference may lie on where the partner cells are. What is the N value of cells observed in WT and rsr1Δ cells? 1% is a low value, so it is likely to be rare. To conclude that it is significantly different, a large number of cells need to be studied.

Minor comments:

Please limit the use of abbreviations such as CS, DS and CKS, PS. It greatly complicates the reading of the manuscript.

Figure 1: Some of the signals are very weak (Sst2-GFP), could you please provide additional examples? The authors follow the localization of GFP-tagged reporters during the formation of "randomly selected zygotes". How was selection of zygotes made? What makes it random?
Please indicate N values of number of cells analyzed.
Please indicate SD not SEM.

Figure 2: What do the green error bars represent? This is not stated in the legend and is unlikely to be the standard deviation.

Figure 3: Please report SD, not SEM, which is meaningless without N value.

Figure 5: The author should provide faster timelapse acquisition to test whether Sec3 and receptor localizations are permanently coincident or not.
It is again unclear what error bars represent. SEM is inappropriate.

Figure 6: The authors should simplify both their schematics and description in the text.

Figure 6, 7 and related supplementary figures: some figures are mis-labeled in the text.

Page 22: I don't see evidence showing that "the Ste2-GFP signal steadily increases", as claimed in the text.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Wang and colleagues present evidence that chemotropic growth during yeast mating occurs via a pheromone tracking mechanism. In this system, pheromone receptors, polarity proteins, and components of the downstream signaling pathway first accumulate at an initial polarity site, which is established adjacent to the zone of cytokinesis. Subsequently, components of the mating response track toward the point of highest pheromone concentration, where they establish a site for chemotropic growth toward their mating partner prior to fusion and zygote formation. This tracking mechanism appears to rely on differential receptor activation, directed secretion at the leading edge of the polarity patch (toward the pheromone gradient), and endocytosis at the trailing edge of the patch. In addition, the authors use computational approaches to model gradient tracking; their model accurately recapitulates a number of their observations (i.e., "pausing" of the tracking machinery at the initial polarity site before migration toward the pheromone source) that are not explained by previous models of this process.

Overall, this study presents intriguing findings that likely are relevant to gradient sensing and chemotropic growth seen in other cell types, and raise the important possibility that gradient tracking mechanisms may be a common theme in polarized growth. Inclusion of a computational model that accurately reflects their observations of yeast mating, and which may be adapted to explain similar processes in other systems, is a clear strength of the study, which should be of interest to the readership of JCB. That said, there are a number of places where the data could be

strengthened and/or the manuscript could be modified for clarity.

Major points:

1. The ability of mating yeast cells to sense a shallow pheromone gradient is undeniably important for their ability to polarize receptors and signaling components, and for shmoo formation toward the source of pheromone in mating mixtures. While surface and cytoplasmic proteins are clearly important, it seems reasonable that an additional factor involved in gradient sensing for MATa cells is the secreted Bar1 protease, which degrades alpha pheromone. It seems plausible that Bar1 might "sharpen" the gradient by limiting the amount of pheromone that engages with surface receptors. If so, one might expect that bar1Δ cells would show a delay in gradient sensing/tracking compared to BAR1+ cells in mating mixtures. The strains used in this study are all BAR1+, but have the authors performed these experiments in bar1Δ cells? Can the computational model predict the effect of bar1Δ on sensing/tracking?

2. Based on the images provided in Fig. 1D, tracking of Sst2-GFP is not overly convincing. The main issue is that the high level of cytoplasmic Sst2 makes it very difficult to discern surface concentration of the protein (e.g., at 10', 20', 50' and 60', the arrow does not appear to mark an area of Sst2 surface accumulation). Polarization is somewhat clearer in Fig. S1, but the authors should consider replacing the cells shown in Fig. 1D.

3. In calculating the rates of Ste2 and G-beta tracking (Fig. S2), the authors report no significant difference in rate for the receptor and G protein in panel C; however; this is less clear when looking at the plots in panels A and B. The individual plots for Ste2 and G-beta have different x- and y-axis scales, but when the data from one plot is transposed to the other, there appears to be a clear difference in slope for the regression analyses that might be interpreted as a difference in tracking rate. By the description in the legend, the plots in panels A and B are representative, presumably of a single trial for each protein. The rates not only differ from each other, but they also differ from the mean reported in the legend and panel C. It would be helpful to show mean tracking behavior in panels A and B for the populations analyzed, rather than representative plots.

4. In Fig. 4B, it is not immediately clear when the cell shown has completed cytokinesis. For other panels in this paper, there is a clear zone of membrane at the boundary between the two cells at 0', but for this panel it looks like there is continuity between the cytoplasm of both cells at 0' and 5'. The accompanying text (p.10) states that G-beta accumulates at the bud neck prior to CK, and then fails to relocalize to the DS, so maybe this is the point that the images are intended to convey. However, the figure legend states that the indicated time frame follows CK. This needs to be clarified.

5. Mutational analysis of the Ste2 C-terminal tail (7XR and 7XR/6SA mutants) is used in Fig. 4 to show that Ste2 phosphorylation is requires for G-beta tracking, while internalization is not. These experiments utilize mutants previously published in Ismael et al. (2016). G-beta still tracks in the 7XR mutant, which cannot internalize, indicating that Ste2 endocytosis is not required for tracking. The authors report that 7XR/6SA cells, which are defective in both internalization and phosphorylation, do not show G-beta tracking, and interpret their results as an indication that phosphorylation is required for tracking. However, an alternative interpretation is that internalization alone is not required for tracking (7XR), but the combination of both internalization and phosphorylation is needed (7XR/6SA). To fully conclude that only phosphorylation is required, the authors need to test the 6SA mutation alone. Otherwise, they should modify the interpretation of their results on p.11 and the corresponding discussion on p.23. The statement in the discussion

that "receptor phosphorylation is both necessary and sufficient for gradient sensing" is not supported by the data: phosphorylation appears to be necessary, but no data, either here or in Ismael et al. (2016) show that it is sufficient.

6. The images in Fig.5A and 5B are not convincing. In the Sec3-RFP panel, gradient tracking occurs counter-clockwise, and Sec3 concentrating in front of Ste2 during tracking is not obvious. Similarly, Sla1 concentrating behind Ste2 is not obvious based on how the data are displayed. The main point of confusion is that arrows are not pointing to the same place on the cell surface in the Ste2 and Sec3/Sla1 panels for each experiment. Simply comparing panels makes it seem like the proteins do not track the same way they appear to in the accompanying plots.

7. While the computational aspect of this paper nicely recapitulates the in vivo observations, the way the model is presented may not be intuitive, and therefore accessible to people who are unfamiliar with computational/modeling approaches. A number of points are not explained or demonstrated:
-p.15, lines 6-8: receptor and G protein polarized to DS in a 6:5 ratio - if this was measured in this study, it isn't shown anywhere, and if it was reported elsewhere, the relevant paper should be cited.
-Explanation of the significance for AA, IA, and II receptor dimers and how they factor into the model is lacking.
-p.15, line 19: shifting of active G-alpha at 1' is mentioned here, but not shown anywhere.

Minor points:

8. The CKS (cytokinesis site) acronym is used only a few times, specifically on p.7 and the legend for figure 1. Its similarity to the CS (chemotropic site) acronym, which is used much more often in the manuscript, could create confusion. The authors may wish to simply write "cytokinesis site" in place of the CKS acronym in the few places it is used.

9. Scale bars should be added for all panels with cell images.

10. In figure 2, it would be helpful to add labels indicating which protein is being observed to the right of each row of plots, in addition to including the description in the legend.

11. p.15, line 13: Callouts for Figs. 7D and S4 should be Figs. 6D and S5.

1st Revision - Authors' Response to Reviewers: June 6, 2019

We thank the reviewers for their careful and helpful evaluation of our work. In answering their comments, we believe we have made considerable improvements to the manuscript. The major changes are summarized below, followed by point-by-point responses to the reviewers.

1. All display images except those in revised Fig. 3B have been deconvolved; all signal plots have been enlarged and displayed under the corresponding images.
2. All image analyses are now fully explained in a section added to the M&M; our method for the normalization/averaging of signal plots across multiple cells is illustrated in new Fig. 2A.
3. Original Figures S3 and S5 have been moved to the main text; they are now Figures 5 and 8.
4. Additional quantifications are shown in new figure panels 3F and 4F, and a cytokinesis marker (GFP-Cdc3) has been co-imaged with Spa2-RFP in revised Fig. 3B.
5. A modified version of the computational model was devised to test the importance of receptor dimerization and the output of the "monomeric" model is shown in Fig. 7F.
6. We simplified Fig. 5 (new Fig. 6) and the corresponding legend and text.
7. We replaced the time-lapse images in Figures 1D and 4D as suggested.
8. A supplementary table demonstrating the robustness of the computational model has been added (Table S9).

Reviewer #1 (Comments to the Authors (Required)):

In this paper Wang and co-authors combine imaging data and a mathematical model to describe the assembly, re-positioning and stabilization of pheromone-signaling machinery at polarity sites during the sexual lifecycle of budding yeast cells. The imaging is in line with recent data that cells sense pheromone gradients at a polarized site and there are interesting novel observations, notably the double fusion of rsr1Δ cells with two partners. However, it lacks rigor in quantification, which lower the confidence in the observations. The model proposes differential sensing by receptors within a single polarize patch, but it is extremely complex and the assumptions and robustness are not explained.

In my opinion, the work needs substantial revision before it can be considered for publication.

Major comments:

The imaging data clearly shows localization of the pheromone receptor and associated proteins (Gbeta, Far1 and Sst2) at a mobile patch. The authors however extract considerable additional information, which I think is poorly supported at this time:

1. Site of initial polarization: this should be co-labelled with a stable transmembrane marker of the default site to claim that it is the default site.

> In haploid cells, the axial bud site, aka the default polarity site, is defined by its position: "Haploid **a** and α cells (as well as a/a and α/α diploids) bud in an axial pattern in which both mother and daughter cells select a bud site immediately adjacent to their previous division site" (Bi and Park, 2012. *Genetics*. 191: 347-387). If Figure 1E-F, we show that following cytokinesis, polarity establishment (PE) of the receptor, Gβ, Far1, and Sst2 occurs at the same position in mating cells as the position of the bud site in vegetative cells, relative to the last division site. Moreover, in *bud1*Δ cells, the receptor polarizes at random positions, not at the default site. From this, we can infer that the receptor polarizes to the DS in *BUD1* cells, as Bud1 function is required to the position the axial-bud/default-shmoo site. We also found that after polarizing at random positions in *bud1*Δ cells, the receptor paused for the usual interval before tracking to the CS. This tells us that the GTM assembly

process is required, but that its position can vary. **In other words, the essential process underlying gradient sensing is GTM assembly, not default localization.**

Even given these lines of evidence, we would be willing to use a stable transmembrane marker of the DS to further support our claim of polarization at the default site. However, it not clear that there such a marker exists. Of the well characterized axial landmarks — Axl1, Axl2, Bud3, and Bud4 — only Axl2 is a transmembrane protein, and its level rapidly declines in response to pheromone (Roemer et al., 1996. *Genes Dev.* 10: 777-793). To our knowledge, there is no published evidence that indicates whether or not the peripheral-membrane axial landmarks persist at the DS in pheromone-treated cells.

In several examples, what dictates the choice of the position and the timing is unclear. For instance, in Fig 1 in the daughter cell of the Gbeta timelapse (not marked), the signal seems to be at very similar position at both 0' (marked as cytokinesis) and 5' (marked as polarity establishment).

After cytokinesis, the mother and daughter cells do not necessarily exhibit identical response kinetics. This is not surprising, given that they are not likely experiencing identical gradient conditions and because daughter cells typically spend a longer time in G1. In Fig. 1B, the arrowheads are placed to indicate PE, tracking, and stabilization of Gβ in the mother cell only. We used the same labeling system as Hegemann et al. (2015), wherein dashed arrowheads indicate PE at the default site, closed arrowheads indicate tracking, and filled arrowheads indicate stabilization at the chemotropic site.

2. More generally, it is unclear how the authors define the position at which to put the arrowheads. Is it the center of distribution or the brightest pixel? For instance, in the Ste2-GFP example of Fig 1 at timepoint 55', the arrowhead seems very decentered. This is important to explain how the start of tracking is defined.

We thank the reviewer for finding this omission. In the original manuscript, we neglected to explain the placement of the arrowheads. This has now been corrected in the Fig. 1 legend. As noted above, the type of arrowhead indicates the status of the GTM — assembling at the DS, tracking, or stabilized at the CS. The arrowheads were placed, by eye, based on the corresponding PM-signal plots (originally shown as small insets, but now enlarged and beneath the images). During assembly, the dashed arrowheads point to the peak at the DS (the dashed blue line); during tracking, the closed arrowheads point to the leading peak at the DS (the dashed green line); coincident with stabilization, the filled arrowheads point to the peak at the CS (the dashed red line). The start of tracking is defined strictly by the PM-signal plots. In Fig. 1A, for example, tracking began between the 10' and 15' timepoints, as indicated by the emergence of a leading peak upgradient from the peak at the default site.

3. Timing since cytokinesis: a cytokinesis marker should be used for co-localization. It is currently unclear how the timing of cytokinesis is precisely defined.

The timing of cytokinesis is not important, per se — any reproducible event in the cell cycle could serve as a zero time. For example, reporter arrival time at the DS could be expressed relative to spindle pole body duplication just as well. Nevertheless, to support the use of Spa2 as a marker of cytokinesis, we cited a paper that shows Spa2-GFP stays at the bud neck until the mother and daughter separate (Dobbelaere and Barral, 2004). We also co-imaged Spa2-RFP and a septin marker (GFP-Cdc3) in cytokinetic cells in mating mixtures (revised Fig. 3B).

4. "Pause" of the patch at polarity establishment: In the few figures that provide images of this early

timepoints, the distribution of Far1 and other components do not look completely static. It could well be that patch movements are already happening there (perhaps less directed).

Yes, it's quite possible that the proteins we studied move in a stochastic fashion within the assembly region before tracking begins. According to our model, directed movement of the GTM components upgradient (i.e., deterministic local gradient tracking) begins when GTM assembly is complete (i.e., at the end of the pause); a finding of non-directed movement before tracking begins would not contradict our claims.

To address the reviewer's comment, we looked at every example that showed at least two time points during the pause (indicated by dashed arrowheads) in the revised figures. None of the reporters showed evidence of non-directed movement in this phase. In one case, the receptor (Ste2-GFP) signal increased and spread out in both directions (new Fig. 6A; old Fig. 5A); in another case, the receptor signal consolidated (new Fig. 6B; old Fig. 5B). The GFP-Gβ and Far1-GFP reporters also exhibited the consolidation behavior (Fig. 1B-C). Of course, this is not an in-depth analysis. Developing a more detailed, mechanistic understanding of what happens during assembly is certainly an important goal for future studies.

5. Sequential arrival of proteins at the polarity site: Fig 3 is difficult to evaluate without seeing the imaging data. First, without two-color imaging, it is difficult to relate differential timings. In addition, I fear that this presented order of events is greatly influenced by the signal to noise levels of the various fluorescent reporters studied. For instance, the Sst2 cytosolic pool shown in Fig 1 appears very large, which likely precludes identification of small cortical signals, which may appear sooner than is reported in this analysis. Similarly, if receptor localization depends on new expression, the folding rate of GFP will influence the timing of detection. Thus, I am not sure that these differential timings of detection represent a real ordered arrival at the polarity site.

We agree with the reviewer that there are some inherent limitations to what we can conclude about the order of arrival based on the assays we describe. It might be, for example, that the receptor (Ste2-GFP) begins to polarize at the DS before Gβ (GFP-Ste4), but its level remains below our detection threshold longer than Gβ. To allow for this possibility, we have made some small yet significant changes to the text. First, we have changed the title of the relevant results section from, "Far1 is the first to **polarize** at the default site…," to "Far1 is the first to **appear** at the default site…," thereby allowing for the possibility that polarization of these reporters might occur in a different order than their detectable appearance at the DS. Second, we added a few words to emphasize that the PE for a given reporter is based on when its signal is first detectable, again allowing for a difference between the measurement and the actuality (see the first sentence of the 2nd paragraph in this section). A second inherent limitation is the signal-to-noise ratio. The reviewer correctly points out that a low signal-to-noise ratio, as in the case of the Sst2-GFP PM/cytoplasm ratio, could adversely affect how precisely we measure PE. This is why we used the ImageJ quantifications and corresponding plots of the signals along the PM, and not the images, to define PE when necessary. We appreciate the reviewer's request for a clarification, as this was not explained in the original manuscript. We have therefore elaborated our protocol for generating these plots and determining PE in a section added to the Materials and Methods, "Image analysis." Finally, we have substantially increased the signal-to-noise ratio of all the displayed images using the Huygens Essential wide-field deconvolution software.

Answers to the other points raised by the reviewer here are as follows:

"*Fig 3 is difficult to evaluate without seeing the imaging data.*"

Although we don't show the imaging data from the experiments represented in Fig. 3, the images shown in Fig. 1 serve as a proxy. Because the spatiotemporal behaviors of the reporters were imaged and quantified in exactly the same way in the two experiments (with the exception of the intervals between time points), we thought that leaving out the images used to generate the data shown in Fig. 3 would be an acceptable space saver. We could certainly add representative images to Fig. 3 if that is deemed necessary. As noted above, text added to the Materials and Methods explains how we defined PE and the initiation of tracking.

"*… without two-color imaging, it is difficult to relate differential timings*."

We chose to compare the timing of PE and tracking initiation using the same fluorescent tag (GFP) for all four reporters so that the rate of folding and signal intensity per molecule would be constants. Although we agree, in principle, that it would be advantageous to follow two reporters in a single cell, we reasoned that the varying properties of different fluorescent proteins would offset the benefits of two-color imaging for the purpose of establishing relative PEs. We did use two-color imaging when studying spatial relationships — i.e., the relative PM distributions of two reporters (old Fig. 5; new Fig. 6).

"…if receptor localization depends on new expression, the folding rate of GFP will influence the timing of detection."

Since we used the same GFP variant to create all four of the reporters analyzed for Fig. 3, we do not expect differences in folding rate to be a significant factor. However, we agree that the relative amounts of extant and nascent reporter molecules localizing to the DS could affect our estimation of the PE for a given protein. For this reason, we have softened our conclusions regarding the order of arrival. In the revised text, we no longer report exact intervals between the PEs; rather, we claim only that these data suggest a particular sequence of localization to, and tracking from, the DS, and point out that our observations are consistent with known interactions (e.g., recruitment of Far1-Cdc24 by Cdc42, recruitment of Gβ by Far1, and interaction of Sst2 with active Gα and the active-unphosphorylated receptor). It is important to note that, unlike our measurement of PE, we do not expect the determination of tracking initiation (defined as a measurable shift of the PM signal peak upgradient) to be affected by the relative proportion of nascent and extant reporter molecules.

To summarize, we stand by our claims that the data presented in Fig. 3 and the corresponding results section allow for qualified conclusions about the relative order of arrival (PE), confirm the existence of the pause, and provide meaningful tracking initiation times. Together with the genetic evidence indicating that Gβ does not localize to the DS in the absence of Far1-Cdc24 interaction, we believe that we are just justified in making the following statement (see end of the Fig. 3 results section):

"These data support the idea that the cell develops its ability to sense and respond to gradient stimulation via a regulated process in G1, which must be completed before orientation toward a mating partner can begin."

6. Rate of tracking: the text states tracking occurs at a constant rate, but no data is shown to support this claim.

The data, which strongly support this claim, are shown in Fig. S2.

The quantifications performed (Fig 2 and others) do not seem to accurately report the actual data. The images do not represent such peaked distributions. I suspect that the averaging method used skews the

data. This is likely the result of centering distributions on the max, rather than on an independent marker of default and chemotropic sites. Using co-imaged independent markers to align the data would be necessary. In addition, providing kymographs of the cell membrane for several individual cells could help interpretation of the data. It is also unclear what the error bars report. Please make sure you report SD, not SEM, which carries no information regarding biological variation and is un-interpretable without knowing the number of samples.

The normalized and averaged plots (Figures 2 and 6C-F and H) *do* accurately represent the imaging data. The reviewer's suspicion that the data are skewed is understandable, however, given that we did not detail how these plots were generated. In the revised manuscript, we describe how we normalized and averaged the plots in Fig. 2 and new Fig. 6 (old Fig. 5) in the "Image analysis" section of the Materials and Methods, and we have added a panel to Figure 2 that illustrates the method.

Briefly, the normalized and averaged plots shown in Fig. 2 were generated as follows: For each mating cell, the distance tracked (DS → CS) in pixels was consolidated to 20 evenly-spaced points, with the signal value for each point determined by the original curve. Equal-sized PM intervals on either side of the tracking region were treated in the same way, producing a 60-point plot that displays the full DS and CS peaks, normalized for tracking distance. As illustrated in the added panel, Fig. 2 A, this method does not change the shape of the curve. The average plots show the mean value ± SEM at each of the 60 points for 20 cells normalized in this way. The point of this analysis is that the normalized and averaged plots (Fig. 2B) show similar tracking kinetics as the single-cell plots for each reporter (Fig. 1), indicating that the GTM behaves consistently across cells. To clarify this point, we have rewritten the conclusion to the Fig. 2 results section. In reconsidering our reasoning, we realized that this analysis does not allow us to say anything about the kinetics of polarization to the DS; we have therefore removed that claim. We also softened the claim from "demonstrate" to "suggest."

Original conclusion: "These plots demonstrate that polarization to the DS and the kinetics of redistribution to the CS are consistent across cells for all four reporters."

Revised conclusion: "These plots are similar to the corresponding single-cell plots, suggesting that the kinetics of redistribution from the DS to the CS are consistent across cells for all four reporters."

For the two-reporter experiments (new Fig. 6), cell circumferences were normalized (rather than the tracking intervals) and the normalization was to 100 points (rather than 60). After normalization, the mean distribution of the receptor was generated by aligning the leading Ste2-GFP peaks with each other during tracking and with the center of the fusion site at the prezygote stage. This allowed us to compare the distribution of Sec3-RFP and Sla1-RFP to that of the receptor during these two phases.

Answers to the other points raised by the reviewer here are as follows:

"*I suspect that the averaging method used skews the data. This is likely the result of centering distributions on the max, rather than on an independent marker of default and chemotropic sites. Using co-imaged independent markers to align the data would be necessary.*"

As discussed above (see point 1), the axial bud site (aka the default polarity site) is defined by its proximity to the last division site. By this criterion, all four of the reporters represented in Fig. 2 sharply polarize to the DS in mating cells (Fig. 1F). Therefore, aligning the DS peaks of a given reporter serves our purpose of studying the consistency of its *tracking* behavior across cells. In contrast, a hypothetical DS marker would likely vary in its exact position from cell to cell, as does the bud site (Fig. 1F). Aligning the DS peaks with this marker would prevent us from following average reporter tracking across cells. Moreover, as noted above, we do not know of an established

transmembrane marker of the DS that persists in pheromone-treated cells. Like the DS, the CS is an intrinsic marker, recognized as the growth site in chemotropic shmoos. Its position corresponds with the center of the fusion zone in prezygotes. The alignment of the Ste2-GFP, GFP-Gβ, Far1-GFP, and Sec3-RFP peaks with the CS in Fig. 2 and new Fig. 6E was revealed, not predetermined, by our analysis.

"*In addition, providing kymographs of the cell membrane for several individual cells could help interpretation of the data.*"

It is unclear to us what kymographs would add. We would expect kymographs of the DS and CS in a given cell to show a gradual signal decrease at the former followed by an eventual signal increase at the latter — less information than we show in our images and plots.

"*It is also unclear what the error bars report. Please make sure you report SD, not SEM, which carries no information regarding biological variation and is un-interpretable without knowing the number of samples.*"

The Fig. 2 legend now indicates that the error bars are SEM; as originally noted, the number of cells used to generate each plot was 20. The choice of SD vs. SEM depends on what one is trying to show. We are trying to determine the average behavior of each reporter in 20 cells (Fig. 2) as a way of asking whether GTM tracking dynamics are relatively consistent from cell to cell, or in 30 cells (old Fig. 5; new Fig. 6) as a way of asking how the average distributions of Sec3 and Sla1 relate to the average distribution of Ste2. SEM is therefore the better choice, as it reflects a confidence interval for the mean behavior of a cohort of cells: when n > 10, 2 X SEM gives a confidence interval of 95% (Cumming et al., 2007. *J. Cell Biol.* 177: 7-11). In our view, this is more informative than showing the variances in signal intensity at each point across the plots.

(Fig 4) The authors then use far1-H7 and ste2-7xR/6SA mutants, concluding that the localization of Gbeta is distinct in these two mutants. This is confusing: both mutants are reported not to orient growth up the pheromone gradient. Far1-H7 cells are reported to use the default site. The authors claim here that the Gbeta does not localize at all to the default site. This is difficult to evaluate without seeing the shmooing and fusion events.

The source of the reviewer's confusion may be the following statement in the original manuscript, which has now been removed: "As expected, *far1-H7* cells invariably shmooed at the DS, unable to orient toward a partner (Valtz et al., 1995)." This statement is misleading, although not incorrect. What we failed to point out is that in our mating mixtures, *MAT***a** *far1-H7 BAR1* cells seldom shmoo, unlike *MAT***a** *far1-H7 bar1Δ* cells treated with saturating pheromone in culture (Valtz et al., 1995). This point, and others brought up here, are clarified in the revised text (see below). We also quantified the time that GFP-Gβ is detectable at the mother-daughter neck before relocalizing to the DS in pre-morphogenic WT, Ste2-7XR, and Ste2-7XR/6SA cells, or to the polarized growth site in post-morphogenic *far1-H7* cells (see new Fig. 4F). In keeping with the qualifications for the measurement of PE (see point 5 above), we changed the third point of our hypothesis rationale from, "Far1 polarizes to the DS before…," to "Far1 **appears** to polarize to the DS before…."

New text: "In the *MAT***a** *far1-H7* cells, GFP-Gβ polarized to the bud neck in late M and remained there, relocalizing to the polarized growth site only after the emergence of a new bud (90% frequency) or a default shmoo (10%) (Fig. 4, B, E and F). In contrast, GFP-Gβ rapidly translocated to the DS following cytokinesis in wild type (WT) cells, after transient localization to the bud neck (Fig. 1 B; Fig. 4 F). These results indicate that the pre-morphogenic DS-localization of both Far1 and Gβ depend on Far1-Cdc24 interaction and suggest that the DS-localization of Gβ depends on that of

Far1. Conversely, localization of GFP-Gβ to the DS was as robust in cells unable to polarize the receptor as in WT cells (Fig. 4, C-F)."

The image shows spreading of the Gbeta signal, which does not look very different from what is shown in case of the ste2-7xR/6SA mutant in the same figure, in which Gbeta is described to relocalize to the default site. This also strengthens the need for an independent marker of the default site. I would like to see more examples, quantification, and imaging until cell fusion. Moreover, in ste2-7xR/6SA cells, is it really still the default site by the time the cell shmoos or did the signal translocate, as it seems?

We understand why the reviewer would say that the signal is spreading out a bit in the original image of GFP-Gβ in far1-H7 cells at the 30' time point, but this change does not represent localization to the DS. First, the quantification we included in the original figure (Fig. 4E), clearly shows that GFP-Gβ does not localize to the DS in *far1-H7* cells (n ≥ 50 for each strain). Second, the deconvolved image (now the 25' time point due to the deletion of an earlier time point in the sequence) shows no evidence of the GFP-Gβ signal spreading out. The slight widening of the signal may result from a shift in the cell's position following cytokinesis. Third, quantification of the GFP-Gβ bud-neck signal over time (new Fig. 4F) clearly shows that GFP-Gβ remains at the neck of *far1-H7* cells until the initiation of new polarized growth, long after GFP-Gβ translocates to the DS in the WT and mutant receptor backgrounds, strongly supporting our claim.

Answers to other comments: The need for an independent marker of the DS has been discussed above. Imaging until cell fusion is shown for the Ste2-7XR cells, which is the only strain represented in this figure that mates at a detectable frequency under our experimental conditions. The apparent translocation of the GFP-Gβ signal in the Ste2-7XR/6SA cells (original Fig. 4D) was likely due to a shift in the cells' position as they shmooed. To avoid confusion, we've chosen a different representative time-lapse (new Fig. 4D).

(Fig 5) It looks clear that the endocytosis distribution is broader than that of secretion, similar to what has previously been shown during budding. However, Sla1 fluorescence signal is weak and I am not convinced by the very specific statement that endocytosis is at the back of the distribution or surrounding the chemotropic site at fusion time. Plotting myself some of the distributions shown in the images does not immediately recapitulate the distributions shown in the example graphs. Maybe there are normalization issues to the quantification.

Huygens Essential widefield deconvolution of these images greatly increases the signal-to-noise ratio. We believe the images shown in Fig. 6B and the corresponding signal plots support our claim that the majority of Sla1 is distributed behind the leading peak of the tracking receptor, and that Sla1 concentrates around the stabilized receptor peak. However, our claim is more strongly supported by the normalized and averaged plots shown in Fig. 6D and 6F. By extracting the mean behavior of each reporter during tracking and stabilization in 30 cells, as described in detail in the revised manuscript (see Materials and Methods, Image analysis), the noise we see in single-cell analysis is filtered out, revealing the spatial relationships we report.

Model: The model is very complex with very many parameters, with unclear assumptions. There are also issues in bulking Gbeta localization with the site of secretion, which is well known to depend on Cdc42 activity. For instance, McClure et al had shown that the Gbeta co-wanders behind the Cdc42 polarity patch when uncoupled from it rather than with it, showing that these are two distinct entities. The well-characterized positive feedback regulations of Cdc42 are not taken into account. I do not think these can

be ignored. It is also not clear how receptor dimerization (which is introduced out of nowhere) influences the model.

We did not mean to imply that Gβγ directs vesicle delivery by itself, but by positioning active Cdc42 (see new Fig. 7Aii), which, as the reviewer points out, is well known to be an essential determinant of directed secretion. We have clarified this point with text added to our description of the model in the Results (p. 15) and in the Materials and Methods (p. 33).

Revision of model description on p. 15: "Secretory vesicles carrying the receptor and heterotrimeric G protein are targeted to the PM by Gβγ. This postulate is based on the well-established interactions between Gβγ and proteins that bind Sec3 — Cdc42 (via Far1-Cdc24) and Rho1 (Bar et al., 2003; Butty et al., 1998; Guo et al., 2001; Nern and Arkowitz, 1998; Nern and Arkowitz, 1999; Pleskot et al., 2015; Zhang et al., 2001) — and on the observation that Gβ is essential to stabilize the position of the polarity complex (McClure et al., 2015)."

The results reported by McClure et al. (2015) may not be strictly comparable to ours, as they did not study gradient-stimulated cells. Their conclusions were based entirely on experiments in which cells were treated with isotropic pheromone and on computational modeling. Despite this significant difference in methodology, none of McClure et al.'s findings contradict our model. In fact, their primary claim is that polarized Gβγ constrains wandering of the polarity complex via its interaction with Far1-Cdc24, which is consistent with our view that the leading peak of Gβγ (i.e., polarized Gβγ) positions the polarity complex in gradient sensing cells via its interaction with Far1-Cdc24. McClure et al. also show that the mobility of the polarity complex decreases as the isotropic pheromone dose increases. The polarity complex wanders freely in the absence of pheromone but is slowed (constrained) as a function of pheromone dose. Sufficiently high doses induce shmooing at the default site, and the shmoos narrow as the dose is further increased. This dose/response experiment mimics what we observe in cells exposed to physiological gradients: the GTM stops tracking and stabilizes when it reaches the region of highest pheromone concentration. The finding that GFP-Gβ trails the unidirectional movement of Spa2-mCherry in *cdc24-m1* cells is not surprising. In our model, Gβγ positions the secretory apparatus in mating cells via its interaction with Far1-Cdc24. If that interaction is broken, as it is in *cdc24-m1* cells, we would expect the communication of pheromone concentration through the receptor to Cdc42 via the G protein to be broken as well, rendering the cells unable to sense the gradient. Indeed, *cdc24-m1* cells cannot detect pheromone gradients nor orient toward mating partners (Nern and Arkowitz, 1998). In the absence of Gβγ-Cdc24 interaction, GFP-Gβ is expected to trail unidirectionally moving Spa2-mCherry because vesicles carrying nascent GFP-Gβ consistently dock and fuse behind the moving polarity complex, as explained in Dyer et al. (2013).

The positive feedback regulation of Cdc42 has been modeled by other groups (e.g., Goryachev and Pokhilko, 2008. *FEBS Lett*. 582: 1437– 1443). In our computational model, all directed trafficking processes (e.g., actin polymerization, vesicle docking, vesicle fusion, receptor-mediated endocytosis etc.) and players (e.g., Cdc42, Cdc24, formins, the exocyst, ubiquitin, clathrin etc.) are represented as aggregate behaviors downstream (and upstream) of the receptor and G protein (analogous to the combination of the receptor, G protein, and Far1-Cdc24 into one species, RecGEF in McClure et al.). This is because our goal is to model how the GPCR and its G protein interact to affect these processes such that the GTM moves upgradient in a directed fashion. Although we agree with the reviewer that Cdc42 positive feedback almost certainly plays an important role in gradient sensing, it isn't clear to us what value would be added by explicitly modeling Cdc42 feedback — or any of the many complex downstream mechanisms — at this point in our investigation.

We agree with the reviewer's points about receptor dimerization. In the revised manuscript, we explain why we used dimeric receptors in the model (Results, p. 15; Materials and Method, p. 31), and we directly test their importance to the model's performance (p. 17 and Fig. 7F).

To be understandable and useful, the following needs to be made explicit:
1. What are the assumptions of the model?

The postulates on which the model is built are described in the Materials and Methods and in the Supplementary Materials. The relevant sections and tables are now cited in the text when the computational model is introduced (p. 15). This should help the reader find the supporting information.

It is not always clear to us what constitutes an assumption. For example, the model depends on the existence of receptor dimers. Homo- and hetero-oligomerization of the Ste2 pheromone receptor have been well documented and we cite the relevant papers. Almost certainly, our modeling of receptor dimers is a simplification, but we would not call dimerization an assumption. Another example of assumption/simplification is how the model treats diffusion (p. 32):

"All proteins are assumed to diffuse laterally, as only the PM is modeled in this analysis. If the effect of surface curvature is ignored, the diffusion of each molecular species can be obtained from Equation 3 (Table S5)." Obviously, soluble proteins, peripheral membrane proteins, and integral membrane proteins diffuse at different rates. The uniform value we use for diffusion is a simplification.

To answer the question more directly, we list below the critical functional relationships in the computational network that have not yet been directly demonstrated in yeast but which we postulate based on cited studies in yeast and other systems.

1. Active receptors can catalytically activate heterotrimeric G proteins by "collision." See p. 31.
2. Any receptor dimer with an inactive monomer (II or IA) can associate with a heterotrimeric G protein and cause its co-internalization. See p. 31.
3. Interaction between a receptor and its kinase renders the receptor signaling incompetent (i.e., unable to activate G proteins). See p. 32.

2. What are the parameters? Table S7 lists variables and parameters, but at least the right-hand side of the table is not obviously understandable. Since I do not know what all the parameters are (krs, krl, krlm,...), I cannot evaluate the assumptions behind the chosen values.

The parameters are defined in Table S3. This is now referenced in Table S8 (old Table S7).

3. Which parameters are constrained by experiments? Which ones are free?

The parameters that are constrained by experiments (i.e., those based on published values) are indicated in Table S8 (old Table S7).

4. How robust is the model? I am concerned that the previous version of the model was sensitive to a 10% change in Gbeta:receptor ratio. Here, the authors use a 6:5 ratio, which they claim is based on experiments. However, fluorescence measurements can estimate an order of magnitude, but not a precision of 10-20%, especially with weak signals as here. If the model hinges on this specific ratio, this should be taken with great caution.

As shown in new Table S9, the model simulates tracking and polarization across a range of values for key unpublished parameters.

The 6:5 ratio was determined by curve fitting to the experimentally determined plots shown in new Fig. S4 (old Fig. S8). The experimental plots, which were taken from Fig. 2 (Start), represent the normalized and averaged distributions of the receptor and Gβ (n = 20 for each reporter). Using the peak ± SEM values for these plots, we can calculate the 95% confidence interval for the starting Receptor:Gβ ratio to be between 1:1 and 1.6:1. The model simulates tracking across a 0.48-10 range of Receptor:Gβ ratios (new Table S9).

(Fig 7) The double mating phenotype of rsr1Δ cells is very interesting and this example clearly shows double polarization of Ste2. In the other examples, representing 99% of the cells, Ste2 localization is not convincing me of the presence of multiple discrete polarity patches. It does not look very different from the WT situation described in Fig 1. Use of a distinct marker of polarization (Bem1 for instance) may be more convincing. The main difference may lie on where the partner cells are. What is the N value of cells observed in WT and rsr1Δ cells? 1% is a low value, so it is likely to be rare. To conclude that it is significantly different, a large number of cells need to be studied.

Now that the images have been deconvolved, the distinct Ste2-GFP patches are quite apparent in Fig. 9C-E (originally Fig. 7C-E). Therefore, we do not think that repeating this experiment with a Bem1 reporter will yield any additional insight.

Indeed, 1% is a low incidence of zygotes formed by a *MAT***a** *bud1*Δ cell that mated with two *MAT*α *BUD1* cells. Put into context, however, it indicates that *bud1*Δ confers a significant effect on mating fidelity. Here are the relevant numbers: In the experiments from which we selected the representative images shown in Fig. 9C-E, we examined approximately 100 zygotes. Of these, three were formed by double-mating. Because only one of the three was captured in our time-lapse imaging, we decided to conservatively report the incidence of double-mating as ~1%. For the experiments represented in Figures 1, 3, and 5 (old Fig. S3), we examined at least 1000 zygotes formed in WT X WT mating mixtures. Including experiments performed for other projects, we have examined at least 10,000 such zygotes. As we have never observed a double mating in a WT mating mixture, the incidence of this phenomenon must be ≤ 0.01% when both partners are *BUD1* — i.e., about 100X lower than when the *MAT***a** partner is *bud1*Δ. We agree with the reviewer that the exact positions of *MAT*α cells around a *MAT***a** *bud1*Δ cell is likely a key factor in double matings.

Minor comments:

Please limit the use of abbreviations such as CS, DS and CKS, PS. It greatly complicates the reading of the manuscript.

We have replaced CKS with “cytokinesis site.” We would like to spell out “chemotropic site,” “default site,” and “polarity established,” but we need to save space/characters. These abbreviations are each used many times.

Figure 1: Some of the signals are very weak (Sst2-GFP), could you please provide additional examples? The authors follow the localization of GFP-tagged reporters during the formation of "randomly selected zygotes". How was selection of zygotes made? What makes it random?

Space restriction precludes showing additional examples from the Sst2-GFP localization experiments. However, Fig. 2B shows the normalized-average behavior of Sst2-GFP in 20 mating cells. We believe that the reviewer will find the Sst2-GFP signal-to-noise ratio in the displayed images has been greatly improved by deconvolution. Our protocol for selecting zygotes for analysis is now described in the "Image analysis" section of the Materials and Methods.

Please indicate N values of number of cells analyzed.

Our careless omission of the N values for Fig. 1F has been corrected ($n \geq 50$ for all strains and measurements).

Please indicate SD not SEM.

We believe the SEM is the more useful measure for our purposes, as discussed above.

Figure 2: What do the green error bars represent? This is not stated in the legend and is unlikely to be the standard deviation.

Corrected: "The plots show the average distribution ± SEM (light green shadow)…"

Figure 3: Please report SD, not SEM, which is meaningless without N value.

$N \geq 50$ for all strains and measurements, as noted in the legend.

Figure 5: The author should provide faster timelapse acquisition to test whether Sec3 and receptor localizations are permanently coincident or not.
It is again unclear what error bars represent. SEM is inappropriate.

Our claim is not that Sec3 and the receptor are permanently coincident, but rather that the Sec3 peak shifts upgradient relative to the receptor peak at the onset of tracking and that the majority of Sec3 aligns with the leading receptor peak during tracking. The legend of Fig. 6 (old Fig. 5) has been corrected to define the error bars. The use of SEM is defended above.

Figure 6: The authors should simplify both their schematics and description in the text.

We agree and have simplified the problematic panels in original Fig. 6 (new Fig. 7) and the corresponding text.

Figure 6, 7 and related supplementary figures: some figures are mis-labeled in the text.

We have made these corrections and all callouts have been double checked.

Page 22: I don't see evidence showing that "the Ste2-GFP signal steadily increases", as claimed in the text.

We quantified the increase in the Ste2-GFP and GFP-Gβ signals during the pause in 25 cells for each reporter, as described in the new "Image analysis" section of the Materials and Methods. The data supporting this claim are shown in a new figure panel (Fig. 3F).

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Wang and colleagues present evidence that chemotropic growth during yeast mating

occurs via a pheromone tracking mechanism. In this system, pheromone receptors, polarity proteins, and components of the downstream signaling pathway first accumulate at an initial polarity site, which is established adjacent to the zone of cytokinesis. Subsequently, components of the mating response track toward the point of highest pheromone concentration, where they establish a site for chemotropic growth toward their mating partner prior to fusion and zygote formation. This tracking mechanism appears to rely on differential receptor activation, directed secretion at the leading edge of the polarity patch (toward the pheromone gradient), and endocytosis at the trailing edge of the patch. In addition, the authors use computational approaches to model gradient tracking; their model accurately recapitulates a number of their observations (i.e., "pausing" of the tracking machinery at the initial polarity site before migration toward the pheromone source) that are not explained by previous models of this process.

Overall, this study presents intriguing findings that likely are relevant to gradient sensing and chemotropic growth seen in other cell types, and raise the important possibility that gradient tracking mechanisms may be a common theme in polarized growth. Inclusion of a computational model that accurately reflects their observations of yeast mating, and which may be adapted to explain similar processes in other systems, is a clear strength of the study, which should be of interest to the readership of JCB. That said, there are a number of places where the data could be strengthened and/or the manuscript could be modified for clarity.

Major points:

1. The ability of mating yeast cells to sense a shallow pheromone gradient is undeniably important for their ability to polarize receptors and signaling components, and for shmoo formation toward the source of pheromone in mating mixtures. While surface and cytoplasmic proteins are clearly important, it seems reasonable that an additional factor involved in gradient sensing for MATa cells is the secreted Bar1 protease, which degrades alpha pheromone. It seems plausible that Bar1 might "sharpen" the gradient by limiting the amount of pheromone that engages with surface receptors. If so, one might expect that bar1Δ cells would show a delay in gradient sensing/tracking compared to BAR1+ cells in mating mixtures. The strains used in this study are all BAR1+, but have the authors performed these experiments in bar1Δ cells? Can the computational model predict the effect of bar1Δ on sensing/tracking?

> We fully agree with the reviewer's suggestion that Bar1 may sharpen the external pheromone gradient. In fact, we have unpublished data indicating that both *bar1*Δ and a mutant G-protein allele confer a defect in gradient sensing without greatly diminishing mating efficiency, whereas in combination, *bar1*Δ and the mutant G-protein allele are synthetically sterile. Our working hypothesis is that Bar1 is required to steepen the external pheromone gradient while the genetically-identified G-protein function is required to steepen the internal signaling gradient, and that at least one of these mechanisms is essential for chemotropic mating. We hope to publish these observations in a future manuscript. To answer the reviewer's question: Yes, in principal, it's possible to use our computational model to predict the effect of *bar1*Δ on gradient sensing. For example, we could decrease the slope of the gradient in our model and ask whether tracking of the receptor and G protein becomes slower and/or less accurate. Because this is certain to be the case if we make the gradient sufficiently shallow, the results of such simulations will not be meaningful until we have some idea of the degree to which Bar1 affects gradient slope*. It is also possible that Bar1 affects the shape of pheromone gradients — e.g., changing them from linear to exponential. While we agree

that the role of Bar1 in gradient sensing is an important question that should be addressed both empirically and with modeling, it is beyond the scope of this manuscript.

*In fact, we did this in silico experiment when analyzing the robustness of the model. Tracking speed slowed by half when we decreased the front-to-back pheromone differential from 18/12 to 15/12 molecules/μm^3, whereas a gradient of less than 13/12 still induced receptor and G-protein polarity.

2. Based on the images provided in Fig. 1D, tracking of Sst2-GFP is not overly convincing. The main issue is that the high level of cytoplasmic Sst2 makes it very difficult to discern surface concentration of the protein (e.g., at 10', 20', 50' and 60', the arrow does not appear to mark an area of Sst2 surface accumulation). Polarization is somewhat clearer in Fig. S1, but the authors should consider replacing the cells shown in Fig. 1D.

This has been corrected. Deconvolution makes it much easier to see the PM Sst2-GFP signal above the cytoplasmic background. We have also chosen a different cell for Fig. 1D in which the Sst2-GFP tracking distance is longer. The positioning of the arrows is explained in the Fig. 1 legend.

3. In calculating the rates of Ste2 and G-beta tracking (Fig. S2), the authors report no significant difference in rate for the receptor and G protein in panel C; however; this is less clear when looking at the plots in panels A and B. The individual plots for Ste2 and G-beta have different x- and y-axis scales, but when the data from one plot is transposed to the other, there appears to be a clear difference in slope for the regression analyses that might be interpreted as a difference in tracking rate. By the description in the legend, the plots in panels A and B are representative, presumably of a single trial for each protein. The rates not only differ from each other, but they also differ from the mean reported in the legend and panel C. It would be helpful to show mean tracking behavior in panels A and B for the populations analyzed, rather than representative plots.

As the reviewer points out, the plots shown in the original Fig. S2 A-B were not representative. This has been corrected in the revised figure. Moreover, we now indicate which mean tracking-rate data points in panel C correspond with the line plots in panels A-B. The important result is that we get a line for each cell and reporter analyzed when we plot distance tracked over time, indicating a largely steady tracking rate from start (DS) to finish (CS). A second important point is that the receptor and Gβ track at the same rate, as indicated by comparing the mean rates for each reporter in 20 cells (panel C). Although we agree with the reviewer that plots of mean tracking behavior for the population analyzed would be meaningful, it is not obvious to us how to plot average tracking rates when the distance tracked is quite variable and we want to determine whether the rates are constant from start to finish. If we were to create such a plot, the N value would decrease as the distance tracked increased along the x-axis.

4. In Fig. 4B, it is not immediately clear when the cell shown has completed cytokinesis. For other panels in this paper, there is a clear zone of membrane at the boundary between the two cells at 0', but for this panel it looks like there is continuity between the cytoplasm of both cells at 0' and 5'. The accompanying text (p.10) states that G-beta accumulates at the bud neck prior to CK, and then fails to relocalize to the DS, so maybe this is the point that the images are intended to convey. However, the figure legend states that the indicated time frame follows CK. This needs to be clarified.

We agree that it is sometimes difficult to determine when cytokinesis has occurred without a fluorescent marker and have therefore removed reference to it in the legend. In the revised figure, we have added an asterisk to mark when the GFP-Gβ reporter is detectable at the mother-daughter

neck (bud neck), and we determined the mean time it remains there in WT, far1-H7, ste2-7XR, and ste2-7SR/6SA cells (new panel 4F; n ≥ 50). Our conclusion, strengthened by the additional quantification, is stated as follows.

New text: "In the *MAT***a** *far1-H7* cells, GFP-Gβ polarized to the bud neck in late M and remained there, relocalizing to the polarized growth site only after the emergence of a new bud (90% frequency) or a default shmoo (10%) (Fig. 4, B, E and F). In contrast, GFP-Gβ rapidly translocated to the DS following cytokinesis in wild type (WT) cells, after transient localization to the bud neck (Fig. 1 B; Fig. 4 F). These results indicate that the pre-morphogenic DS-localization of both Far1 and Gβ depend on Far1-Cdc24 interaction and suggest that the DS-localization of Gβ depends on that of Far1."

5. Mutational analysis of the Ste2 C-terminal tail (7XR and 7XR/6SA mutants) is used in Fig. 4 to show that Ste2 phosphorylation is requires for G-beta tracking, while internalization is not. These experiments utilize mutants previously published in Ismael et al. (2016). G-beta still tracks in the 7XR mutant, which cannot internalize, indicating that Ste2 endocytosis is not required for tracking. The authors report that 7XR/6SA cells, which are defective in both internalization and phosphorylation, do not show G-beta tracking, and interpret their results as an indication that phosphorylation is required for tracking. However, an alternative interpretation is that internalization alone is not required for tracking (7XR), but the combination of both internalization and phosphorylation is needed (7XR/6SA). To fully conclude that only phosphorylation is required, the authors need to test the 6SA mutation alone. Otherwise, they should modify the interpretation of their results on p.11 and the corresponding discussion on p.23. The statement in the discussion that "receptor phosphorylation is both necessary and sufficient for gradient sensing" is not supported by the data: phosphorylation appears to be necessary, but no data, either here or in Ismael et al. (2016) show that it is sufficient.

We fully agree and thank the reviewer for finding this mistake. At present, we know of no way to prevent receptor phosphorylation without blocking internalization. Therefore, we have modified the claim in the Discussion (p.23) of the revised text. Note that we have not changed the original claim in the Results (p.11), as it is correct: "These results demonstrate that Gβ tracking from the DS to the CS requires receptor phosphorylation, but not receptor ***polarization***." (Emphasis added.) The point here is that neither the ste2-7XR nor the ste2-7XR/6SA mutant receptors polarize, whereas tracking occurs in ste2-7XR but not ste2-7XR/6SA cells.

6. The images in Fig.5A and 5B are not convincing. In the Sec3-RFP panel, gradient tracking occurs counter-clockwise, and Sec3 concentrating in front of Ste2 during tracking is not obvious. Similarly, Sla1 concentrating behind Ste2 is not obvious based on how the data are displayed. The main point of confusion is that arrows are not pointing to the same place on the cell surface in the Ste2 and Sec3/Sla1 panels for each experiment. Simply comparing panels makes it seem like the proteins do not track the same way they appear to in the accompanying plots.

We have flipped all the images in panels A and B of Fig. 6 (old Fig. 5) so that the tracking direction is clockwise (as in the corresponding signal plots) and the positioning of the arrowheads is now described in the figure legend. The display images have been deconvolved in the revised manuscript, which makes it much easier to see weak PM signals. It is important to note that the image analysis (now detailed in the Materials and Methods) was performed on the raw data. In some cases, this may result in a poorer correspondence between the plots and the display (deconvolved) images. Nevertheless, we believe the spatial relationship between Sec3-RFP and Ste2-GFP is quite clear in both the single-cell time-lapse images and plots shown in Fig. 6A. On the other hand, we agree with

the reviewer that the spatial relationship between Sla1-RFP and Ste2-GFP is harder to see in the single-cell time-lapse images and plots shown in Fig. 6B due to the punctate localization of the Sla1 reporter (which may reflect the clustering of receptor internalization). Examination of many such single-cell time-lapse images supports our claim that the bulk of Sla1-RFP is behind the leading Ste2-GFP peak during tracking and that Sla1-RFP concentrates around the center peaks of receptor and Sec3 just before morphogenesis. However, our claim is more strongly supported by the normalized and averaged plots shown in Fig. 6D and 6F. By extracting the mean behavior of each reporter during tracking and stabilization in 30 cells, as described in detail in the revised manuscript (see Materials and Methods, Image analysis), the noise we see in single-cell analysis is filtered out, revealing the spatial relationships we report.

7. While the computational aspect of this paper nicely recapitulates the in vivo observations, the way the model is presented may not be intuitive, and therefore accessible to people who are unfamiliar with computational/modeling approaches. A number of points are not explained or demonstrated:
-p.15, lines 6-8: receptor and G protein polarized to DS in a 6:5 ratio - if this was measured in this study, it isn't shown anywhere, and if it was reported elsewhere, the relevant paper should be cited.
-Explanation of the significance for AA, IA, and II receptor dimers and how they factor into the model is lacking.

We appreciate the reviewer's feedback on the description of the model and identification of points that require elaboration. We hope that the additions we've made to the revised manuscript will satisfy his/her concerns. Please see our responses to reviewer #1's comments regarding the model.

-p.15, line 19: shifting of active G-alpha at 1' is mentioned here, but not shown anywhere.

Our claim is that in the simulation, active G-alpha shifts upgradient at 3'. This is shown in Video 2; it is best seen by stepping through the video one frame at a time. The callout is in the second sentence of this paragraph: "Figures 6 C and S5, and Videos 1 and 2 show selected outputs of the model, beginning with the receptor and G protein already polarized at the "DS."

Minor points:

8. The CKS (cytokinesis site) acronym is used only a few times, specifically on p.7 and the legend for figure 1. Its similarity to the CS (chemotropic site) acronym, which is used much more often in the manuscript, could create confusion. The authors may wish to simply write "cytokinesis site" in place of the CKS acronym in the few places it is used.

We agree and have made this change.

9. Scale bars should be added for all panels with cell images.

Scale bars have been added to all of the DIC images.

10. In figure 2, it would be helpful to add labels indicating which protein is being observed to the right of each row of plots, in addition to including the description in the legend.

Done. We added labels to indicate which reporter was being followed at the far left of each plot.

11. p.15, line 13: Callouts for Figs. 7D and S4 should be Figs. 6D and S5.

We have made these corrections and all callouts have been double checked.

June 28, 2019

RE: JCB Manuscript #201901155R

Dr. David E Stone
University of Illinois at Chicago
Biological Sciences
Molecular Biology Research Building
900 South Ashland
Chicago, Illinois 60607

Dear Dr. Stone,

Thank you for submitting your revised manuscript entitled "Mating yeast cells use an intrinsic polarity site to assemble a pheromone-gradient tracking machine". You will see that both referees find the conclusions more solid and are in favor of further consideration at the journal. However, Rev#1 requests a few final changes - several of which could be addressed in the text and/or by showing other examples of the data. The reviewer requests more clarity on the definition of the default site, strengthening of the pausing claim (points #2 and #3) and two-color imaging (point #1) regarding the timing of arrival of proteins. These points seem consistent to us with the points they raised in the first round of review and we agree that these final concerns need to be resolved definitively for publication to ensure that the conclusions are clear and robust.
We would be happy to publish your paper in JCB pending changes to thoroughly and definitively address Rev#1's final comments and final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please provide an eTOC statement on the manuscript title page at resubmission, starting with "Wang, Tian, et al...." to match JCB's preferred style.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the

text for readers who may not have access to referenced manuscripts.
- More info about Molecular and microbiological techniques - even if described previously in other work
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

5) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include ~1 sentence per item.

6) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

7) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Ian Macara, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this revised manuscript, the authors have significantly improved both the quality of their images and the description of their model. I think that the idea that the polarity patch moves in a deterministic manner towards the source of the pheromone gradient is an interesting one, which the data largely supports. However, there are a few points, where I remain unconvinced.

1. Given the small differences in timing between the various components of the GTM, I remain completely unconvinced that one can cross-compare without a common marker, as shown in Figure 1. The authors have done the work of using a common Spa2-RFP reference in all their strains in Figure 3. The paper would be significantly improved by showing in Figure 1 the images corresponding to the timing analysis of Figure 3, where Spa2 would provide both positional and timing cross-reference across the strains. This does not require more experimental work.
2. The pausing phenomenon remains poorly described in my eyes, as it is principally shown by 1 (Fig 1A) or 2 timepoints (Fig 1B-C-D) in time-lapse imaging, for which the profile is not always provided (absent in Fig 1D, as well as in Fig 6). Providing higher temporal resolution of this phenomenon would help.
3. Though I understand the author's argument that there is no clear marker of the DS, the definition of the DS remains confusing. In figure 1, the authors explicitly describe it as the location close to the previous division site where the cell would bud, and show quantification of angle to this effect, which is around 35{degree sign}. However, later in the text, two examples of cells are given that shmoo "at the DS", in far1-H7 (fig 4B) and ste2-7XR/6SA (fig 4D), where the shmoo appears to be exactly at the previous division site, which would represent an angle of 0{degree sign}. Therefore,

either the choice of example is very poorly representative of the average (note that in the angle quantification, there is hardly any value below 10{degree sign}), or the DS is a poorly defined concept, or maybe the "default" is the site of division, where assembly of the GTM starts?
4. In figure 5, the two examples chosen to illustrate the imprecision of the ste2-7XR mutant are subject to alternative interpretation. In the top example (5B), two a-cells are polarizing towards the same alpha partner. The overshoot of the top a cell may be due to the alpha cell temporarily polarizing towards the bottom a cell for instance, as has been shown in S. pombe unstable polarity. In the bottom example (5C), the cells are moving relative to each other, so there must be some disturbance on the slide, which likely also causes changes in pheromone gradients. The authors need to provide more convincing examples, where cells are not moving and faced with a single possible partner, or mark the polarity site in the alpha-cell too to show it remains stable when confronted with several partner cells.
5. In the model, because Gbeta-Cdc42-secretion are aggregated together, the condition where the initial distribution of Gbeta is set to uniform, which shows failure of tracking, does not correspond to any tested in vivo situation and does directly support a role for Gbeta polarization before the receptor. It simply indicates that polarization fails if Cdc42/secretion is distributed throughout the cortex. This should be rephrased.

Minor comments:

Figures 3F and S2A-B need error bars.

The phrasing "in cells unable to polarize the receptor" is slightly unclear (top of p. 11). Please replace by more precise "in cells expressing a receptor that is not endocytosed and thus unable to polarize" or similar.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Wang and colleagues present evidence that chemotropic growth during yeast mating occurs via a pheromone tracking mechanism. In this system, pheromone receptors, polarity proteins, and components of the downstream signaling pathway first accumulate at an initial polarity site, which is established adjacent to the zone of cytokinesis. Subsequently, components of the mating response track toward the point of highest pheromone concentration, where they establish a site for chemotropic growth toward their mating partner prior to fusion and zygote formation. This tracking mechanism appears to rely on differential receptor activation, directed secretion at the leading edge of the polarity patch (toward the pheromone gradient), and endocytosis at the trailing edge of the patch. In addition, the authors use computational approaches to model gradient tracking; their model accurately recapitulates a number of their observations (i.e., "pausing" of the tracking machinery at the initial polarity site before migration toward the pheromone source) that are not explained by previous models of this process.

The revised version addresses a number of concerns raised during the initial review, and substantially improves upon the data presented. Specifically, providing deconvolved images for many panels in which there were issues in discerning membrane-associated vs. cytoplasmic fluorescence make these data much more convincing. Moreover, the improved description of the in silico model will make this aspect of the study more accessible to people who are not familiar with these approaches.

My original opinion of this study still holds: that it provides novel insights into tracking of a polarized cue, and that it is relevant and of interest to the readership of JCB. With the revisions provided here, I am satisfied that my previous concerns have been sufficiently addressed.

Dear Dr. Macara and Dr. Casadio,

Thank you for your continued interest in our manuscript, “Mating yeast cells use an intrinsic polarity site to assemble a pheromone-gradient tracking machine.” Please see our responses to Reviewer 1’s comments about the revised manuscript below.

Reviewer #1 (Comments to the Authors (Required)):

In this revised manuscript, the authors have significantly improved both the quality of their images and the description of their model. I think that the idea that the polarity patch moves in a deterministic manner towards the source of the pheromone gradient is an interesting one, which the data largely supports. However, there are a few points, where I remain unconvinced.

1. Given the small differences in timing between the various components of the GTM, I remain completely unconvinced that one can cross-compare without a common marker, as shown in Figure 1. The authors have done the work of using a common Spa2-RFP reference in all their strains in Figure 3. The paper would be significantly improved by showing in Figure 1 the images corresponding to the timing analysis of Figure 3, where Spa2 would provide both positional and timing cross-reference across the strains. This does not require more experimental work.

> The reviewer is conflating the conclusions we drew from Figures 1 and 3. The point of Figure 1 is that the four reporters we studied behaved similarly in mating cells, as summarized in the last sentence of the text describing this figure: “Like the receptor and Gβ reporters, Far1-GFP and Sst2-GFP exhibited dynamic localization patterns: following cytokinesis, they polarized to the DS and paused before incrementally redistributing to the CS, where they stabilized just before shmoo emergence.” We are not claiming to show precise kinetic comparisons of the four reporters in the experiments represented by Fig. 1; we are only giving a general description of how each reporter’s localization changes in mating cells between cytokinesis and fusion with a partner.
>
> In Figure 3, on the other hand, we do compare the times of arrival (PE), pause, and tracking initiation for the four reporters. To make these measurements, we used Spa2-RFP as a reproducible marker of cytokinesis and took 2-minute time points (as opposed to 5-minute time points in Fig. 1). It is important to note that the comparisons shown in Fig. 3C-E are based on at least 50 measurements for each strain and parameter, and that the claimed differences show high statistical significance.
>
> The reviewer’s criticism here echoes his/her original comment, “*Fig 3 is difficult to evaluate without seeing the imaging data.*” In response, we said that we had left the images out to save space but could certainly add them if that was deemed necessary. Indeed, we can add a figure showing representative images of the data underlying the quantitative analyses shown in Fig. 3, but in our opinion, this would not substantively improve the manuscript nor warrant the use of additional space.

2. The pausing phenomenon remains poorly described in my eyes, as it is principally shown by 1 (Fig 1A) or 2 timepoints (Fig 1B-C-D) in time-lapse imaging, for which the profile is not always provided (absent in Fig 1D, as well as in Fig 6). Providing higher temporal resolution of this phenomenon would help.

> The images shown in Figures 1 and 6 are not our only evidence for the pause; we show robust quantification of the pause times in Fig. 3D. As noted above, we used a reproducible time marker, 2-minute time points, and measured the pause time in at least 50 cells for each reporter. Again,

we can include representative images corresponding to the data reported in Fig. 3, but we do not think this would strengthen our claims.

As suggested by the reviewer, we have added signal plots for all the images that show a paused reporter, but which previously lacked the corresponding quantitation (Fig. 1D-20', Fig. 6A-15', and Fig. 6B-10',15').

3. Though I understand the author's argument that there is no clear marker of the DS, the definition of the DS remains confusing. In figure 1, the authors explicitly describe it as the location close to the previous division site where the cell would bud, and show quantification of angle to this effect, which is around 35{degree sign}. However, later in the text, two examples of cells are given that shmoo "at the DS", in far1-H7 (fig 4B) and ste2-7XR/6SA (fig 4D), where the shmoo appears to be exactly at the previous division site, which would represent an angle of 0{degree sign}. Therefore, either the choice of example is very poorly representative of the average (note that in the angle quantification, there is hardly any value below 10{degree sign}), or the DS is a poorly defined concept, or maybe the "default" is the site of division, where assembly of the GTM starts?

Both the axial bud site (where haploid cells bud) and the default polarity site (where cells treated with isotropic pheromone shmoo) are simply and unambiguously defined as proximal to the last division site. There is no disagreement about this in the field. As shown in Fig. 1F, we found the angle between the middle of the division site and the incipient bud site to have a mean ± SEM of 33.7 ± 0.9˚ (n ≥ 50); the positions of PE for the receptor, Gβ, Far1, and Sst2 reporters were indistinguishable.

The reviewer's confusion seems to result from two mutant cells that s/he says shmoo at the division site, rather than at the DS. The first example is the *MAT***a** *far1-H7* mutant cell shown in Fig. 4B. Although this cell does elongate a bit during the 25' time-course, it is not clear to us that it shmooed, nor whether it is elongating at its division site or its default site. As we pointed out in our first response letter, *MAT***a** *far1-H7 BAR1* cells seldom shmoo in mating mixtures. The other example is the *ste2*$^{7XR/6SA}$ cell shown in Fig. 4D. The important conclusion we draw from the experiment represented by this panel is that in *MAT***a** *ste2*$^{7XR/6SA}$ cells, GFP-Gβ localizes to the DS but is unable to track upgradient. Fig. 4D shows time-lapse images of a mother and daughter *MAT***a** *ste2*$^{7XR/6SA}$ pair expressing the GFP-Gβ reporter. Following cytokinesis, the reporter clearly localizes to the DS in both the mother and daughter but fails to track upgradient. Our conclusions about the initial localization of GFP-Gβ (i.e., its movement from the mother-daughter neck to the DS) in these mutant cells is strongly supported by the data shown in Fig. 4E-F. We agree with the reviewer that the mother cell in Fig. 4D appears to shmoo at the division site — perhaps because the cell is expressing a mutant form of the receptor that cannot be phosphorylated or internalized — and that this is unusual. Therefore, we have added another representative time-lapse to this panel. However, we do not agree that the aberrant shmoo site in the original panel calls into question the definition of the default site, nor the inferences we drew from this experiment.

4. In figure 5, the two examples chosen to illustrate the imprecision of the ste2-7XR mutant are subject to alternative interpretation. In the top example (5B), two a-cells are polarizing towards the same alpha partner. The overshoot of the top a cell may be due to the alpha cell temporarily polarizing towards the bottom a cell for instance, as has been shown in S. pombe unstable polarity. In the bottom example (5C), the cells are moving relative to each other, so there must be some disturbance on the slide, which likely also causes changes in pheromone gradients. The authors need to provide more convincing examples, where cells are not moving and faced with a single possible partner, or mark the polarity site in the alpha-cell too to show it remains stable when confronted with several partner cells.

We do not agree with the reviewer's reasoning on this point (see rebuttal below). Nevertheless, we agree that additional examples of under- and over-tracking could strengthen our claim, and we have therefore included a second representative time-lapse in both Figures 5B and 5C.

The reviewer suggests that the overshooting behavior of the tracking *MAT***a** *ste2*^{7XR} cell shown in Fig. 5B could be due to transient polarization of its *MAT*α partner towards the *MAT***a** *ste2*^{7XR} cell beneath it. This is unlikely. We would expect polarization of the *MAT*α cell towards the lower *MAT***a** *ste2*^{7X} cell to shift the gradient such that the upper *MAT***a** *ste2*^{7XR} cell would under-track, not over-track its target. Moreover, we observed over- or under-tracking in 13 of the 20 *MAT***a** *ste2*^{7XR} cells we examined (see Fig. 5 legend), whereas we almost never see such phenotypes in WT mating mixtures.

In Fig. 5C, the reviewer infers that there must have been some disturbance on the slide because the mating cells appear to change positions over the course of the time-lapse. Although we cannot definitively eliminate this possibility, we think a more likely explanation is that the mating partners pushed each other into new positions as they began to shmoo. This is not uncommon. In Fig. 1D, for example, the *MAT*α cell shifts position downward with respect to its mating partner. As we almost invariably see in WT X WT crosses, however, the *MAT***a** cell tracks the movement of its partner and fuses with it straight on (i.e., zygote fusion angle = 0˚). Given that WT cells fuse at a mean angle near zero (Ismael et al., 2016), the incidence of tracking anomalies in WT cells that result in angled zygotes must be small. Therefore, we are certain that the high incidence of tracking anomalies we observe in *MAT***a** *ste2*^{7XR} cells is due to the mutant receptor, not to technical glitches.

5. In the model, because Gbeta-Cdc42-secretion are aggregated together, the condition where the initial distribution of Gbeta is set to uniform, which shows failure of tracking, does not correspond to any tested in vivo situation and does directly support a role for Gbeta polarization before the receptor. It simply indicates that polarization fails if Cdc42/secretion is distributed throughout the cortex. This should be rephrased.

The reviewer has misstated the result of the in-silico experiment shown in Fig. 7E. Setting the initial condition of the computational model such that the receptor is polarized and the G protein is uniformly distributed did not cause polarization to fail. In fact, both the receptor and G protein polarized robustly, directly, and rapidly to the chemotropic site, taking one third the time that the standard model and the "polarized G-protein/uniform receptor" version of the model took to track to the CS. In other words, starting with uniform G protein results in a global gradient sensing behavior, completely unlike the GTM assembly and tracking phenomena we observe in vivo. As long as the G protein starts off polarized, however, the model mimics the deterministic local gradient sensing property of the GTM. Thus, the simulations shown in Fig. 7D-E correlate with our experimental observation that Gβ polarizes to the DS before the receptor.

In summary, it seems to us that reviewer 1 has carefully and fairly examined the representative images but has undervalued the quantified population data.

Minor comments:

Figures 3F and S2A-B need error bars.

We have added error bars to Fig. 3F. Error bars are not applicable to Figure panels S2A-B because these plots represent the measured values of tracking speed for single cells. There is only one value for each time point for a given cell.

The phrasing "in cells unable to polarize the receptor" is slightly unclear (top of p. 11). Please replace by more precise "in cells expressing a receptor that is not endocytosed and thus unable to polarize" or similar.

We changed this phrase to, “in cells unable to internalize and thereby polarize the receptor.”