# Cega: A Single Particle Segmentation Algorithm to Identify Moving Particles in a Noisy System

Erin Masucci, Peter Relich, E. Ostap, Erika Holzbaur, and Melike Melike Lakadamyali

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-11-0744
TITLE: Cega: A Single Particle Segmentation Algorithm to Identify Moving Particles in a Noisy System

Dear Prof. Melike Lakadamyali:

Thank you for the interesting submission. Please revise according
to the comments of two reviewers, and I will send the manuscript to
one of the reviewers for the second look.

Sincerely,

Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell


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Dear Prof. Melike Lakadamyali,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org


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Reviewer #1 (Remarks to the Author):

In this work, Masucci et al. develop an approach "Cega" for the detection of mobile particles in noisy images that are otherwise challenging to detect using only direct intensity information in the image. The fundamental idea is to use temporal information

over multiple frames to gather evidence for the existence of dim, mobile particles (in a way similar to what a human observer would do).

In brief, first the images are converted from arbitrary units to photons (by calibrating the camera) in order to get Poisson-like signal statistics. Then the images are processed to highlight mobile particles and suppress background noise, including stationary particles, which are considered part of experimental noise for the purposes of their analysis. Mobile particles are highlighted by performing a spatiotemporal convolution of the movie according to a ballistic diffusion model. Background noise (including stationary particles) is highlighted by performing an equivalent convolution but using a stationary model. The difference between the "mobile movie" and the "stationary movie" is then calculated using the Kullback-Leibler divergence. Roughly speaking, regions where the difference is large contain mobile particles, while regions where the difference is small do not. Thus, detection is performed on the Kullback-Leibler divergence "image" (after some further processing to eliminate spurious differences).

The approach is smart and it is clearly useful for the authors' purposes. Nevertheless, the work and manuscript can be improved in terms of rigor and clarity.

My main concern is that the authors' justifications for the various parameter values in Cega are often qualitative and without explicit evidence (e.g. figures or tables to show data). For example, on p. 8 the authors state "We chose a sliding window of 31 frames, 15 frames before and after the pixel of interest to sample our median pixel as it appeared to provide the best compromise between dynamics suppression and background estimation accuracy." The authors should show this compromise explicitly.

The connectivity filter (3 pixels and 0,1 nat) and LoG filter (3x3 area and 5 nats) parameters also seem quite arbitrary.

Just to be clear, these are only examples, and are not a comprehensive listing.

Along the same lines, some of the wordings in the manuscript are quite subjective. For example, in the "offset and gain calibration" part of the Methods, the authors state "we performed some modifications to existing software to return reasonable calibration parameters" (p. 19). What are these modifications, and what is "reasonable" about the calibration parameters?

First line on p. 11, the authors write "softened version of the calibrated movie." What exactly does "softened" mean?

More importantly, because of this issue of qualitative and subjective statements, how is a user of this algorithm to decide what parameter values to use? It would be useful if some of the spatiotemporal convolution filter parameters can be related to the expected movement of the particles for example.

In Fig. 1C, the LoG row: what are the white lines and pixels around the circles indicating the objects? Why are they so prominent? They are not there/barely there in the "connectivity" images.

In the subsection entitled "tracking" on p. 13, the authors do not mention what tracking algorithm they use. Instead, they introduce it in the discussion (bottom of p. 16). This information should go up to the tracking section of the results.

Also, what SNR was used for the tracking tests? This does not seem to be mentioned anywhere.

For the tests with simulated data, i.e. Figs 2 and 3, what is the sample size? It would be good to show a mean and standard deviation per photon count in Figs 2 and 3.

For the sake of clarity, the authors should not interchange the terms "detection" and "tracking." For example, at the bottom of p. 12, the authors state "No algorithm tested was able to provide a tracking solution at 50 photons." What the authors really mean is detection. Of course no detection means no tracking, but there is no need to confuse the two terms.

For completeness, the authors should comment on whether/how a similar approach can be pursued if not only dim mobile objects are of interest, but also dim stationary objects. Often, the objects of interest are not mobile all the time.

Regarding the manuscript, its organization can be greatly improved. The "computational strategy" section does not cite any figures, even though there are relevant figure panels, which are then cited in the following section entitled "characterization/optimization of performance." This latter section feels rather repetitive with the computational strategy section, and as far as I can see it does not contain any characterization or optimization of performance. It is primarily a simplified, non-mathematical summary/repeat of the computational strategy section.

There are 8 videos associated with this manuscript, but they are not cited as far as I could see.

Reviewer #2 (Remarks to the Author):

The authors present an improved particle tracking method Cega to study single molecule movement in noisy biological systems. They have both tested the method on simulated and real data with excellent results. This is an important contribution to the community and will aid biological research in particular in studying cell biology and signaling processes. The authors have made the software available for use by other researchers.

Comments

1. The authors mention the use of EMCCD cameras, I wonder how the algorithm would perform when using sCMOS type of cameras that display aberrant hot pixel fluctuations that can affect noise statistics in a detrimental way.

2. Background may be structured or vary over time and not per se uniform, the authors use a temporal kernel. For Stochastic Super resolution microscopy techniques temporal (median) filters have been used efficiently and successfully. The authors did not discuss or elaborate much on this. These SSRM studies also show that structural background other give more artifacts, did the authors systematically test this for their approach?

3. Fluorophores can have very different photon counts, especially fluorescent proteins are much dimmer than organic dyes, this in combination with specific uniform or structured background levels may be critical for performance, it is not clear if this was also explored in detail, hence increasing background levels with specific photon counts per event.

4. The authors included videos, however are not clearly cited/described in the text.

5. I would highly recommend to make all real and simulated (with generation scripts) data available, for example via Zenodo with a DOI.

We thank the reviewers for their thoughtful comments and constructive suggestions, which helped us improve our manuscript. We have addressed the reviewer comments in the manuscript and marked the revisions with highlighted text. In addition, we provide below a point by point response to each reviewer comment. The comments are in black text and our response is in blue text.


Reviewer #1 (Remarks to the Author):

In this work, Masucci et al. develop an approach "Cega" for the detection of mobile particles in noisy images that are otherwise challenging to detect using only direct intensity information in the image. The fundamental idea is to use temporal information over multiple frames to gather evidence for the existence of dim, mobile particles (in a way similar to what a human observer would do).

In brief, first the images are converted from arbitrary units to photons (by calibrating the camera) in order to get Poisson-like signal statistics. Then the images are processed to highlight mobile particles and suppress background noise, including stationary particles, which are considered part of experimental noise for the purposes of their analysis. Mobile particles are highlighted by performing a spatiotemporal convolution of the movie according to a ballistic diffusion model. Background noise (including stationary particles) is highlighted by performing an equivalent convolution but using a stationary model. The difference between the "mobile movie" and the "stationary movie" is then calculated using the Kullback-Leibler divergence. Roughly speaking, regions where the difference is large contain mobile particles, while regions where the difference is small do not. Thus, detection is performed on the Kullback-Leibler divergence "image" (after some further processing to eliminate spurious differences).

The approach is smart and it is clearly useful for the authors' purposes. Nevertheless, the work and manuscript can be improved in terms of rigor and clarity.

1. My main concern is that the authors' justifications for the various parameter values in Cega are often qualitative and without explicit evidence (e.g. figures or tables to show data). For example, on p. 8 the authors state "We chose a sliding window of 31 frames, 15 frames before and after the pixel of interest to sample our median pixel as it appeared to provide the best compromise between dynamics suppression and background estimation accuracy." The authors should show this compromise explicitly.

The connectivity filter (3 pixels and 0,1 nat) and LoG filter (3x3 area and 5 nats) parameters also seem quite arbitrary.

Just to be clear, these are only examples, and are not a comprehensive listing.


We agree with the reviewer's assessment. Accordingly, we have included an explanation of how we calculated out these parameters in the text in the "Computational Strategy and Optimization" section pgs 5-11 and a guide for how these values can be calculated from other data in Table 1 on pg 27 line 671.

   Pg 8 lines 186-188: "We chose a spatiotemporal window blur of 5 frames (2 frames before and after) since > 95% of the moving motor signal in our data is maintained within 2 adjacent pixels."

Pg 9 lines 207-214 "Ideally, the median filter needs to suppress dynamical fluctuations from moving motors while representing a gradually fluctuating background as accurately as possible. Therefore, the sliding window must exceed the duration of the moving motor signal, or else the motor signal will remain within the stationary model and will be removed when the KL divergence is applied against the motion model. We chose a sliding window of 31 frames, 15 frames before and after the pixel of interest to sample our median pixel as it appeared to provide the best compromise between dynamics suppression and background estimation accuracy (Figure S1 and S2). For our data, >95% of moving motors moved within 31 frames."

Pg 10-11 lines 247-252 "The spurious noise of the KLM is removed with a connectivity filter (Table 1, Figure 1C, Connectivity, and Video 1-4), which eliminates all but the top 95% of the data within this model that includes the moving motor signal (Figure S1 and S2). To do this, any 3x3 pixel subregion in the KLM must have at least 3 pixel values greater than the 95th quantile, measured to be 0.1 nats (units of natural logarithm), or the center pixel of that subregion is set to 0 in the connectivity movie."

Pg 11 lines 269-271 "We used the 95th quantile of the connectivity movie (5 nats) to preserve only coordinate positions of moving motors (Figure S1 and S2)."

Table 1. Cega thresholding values used for experimental data.

| Step | Optimal values estimated for experimental data | How to estimate values | Additional considerations for optimal use |
|---|---|---|---|
| Pixel calibration | Offset = 2293 & Gain = 71 | Estimated from series of dark frames from EMCCD camera (see Methods). | Data must be calibrated before Cega. Calibration is specific to the camera used. |
| Stationary model estimation | Sliding temporal window median filter = 31 frames. | Duration of >95% of moving motors of interest. | If tracking particles that move for long periods of time or periodically pause, choose a window size encompassing the duration of >95% of the particles of interest. |
| Motion model estimation | Spatiotemporal gaussian blur = 5 frames | Duration that >95% of moving motors remain within 2 adjacent pixels. | Smaller temporal kernel provides better computational efficiency. |
| KL divergence | No user defined parameters are required. | No user defined parameters are required. | Stationary and motion models are used as input. |
| Connectivity Filter | 3 connected pixels > 0.1 nats | Threshold set to 95th percentile of connectivity model. | Additive salt noise results in many false positives. This noise is removed before candidate finding by applying a threshold. |
| LoG Filter | 3x3 neighborhood pixel sum from | Threshold set to 95th percentile of LoG model. | The LoG image sequence is used to find initial local minima. Then, connectivity image sequence values for these |

| | connectivity filter > 5 nats. | | positions are used to threshold local minima appropriately. |

In addition, we performed a parameter sweep of the stationary model sliding window, connectivity filter threshold and LoG filter threshold indicating that the chosen parameters are optimal for our datasets and included this information in a set of supplementary figures (Figure S1-S3). Cega's performance was more sensitive to the stationary model sliding window size and the LoG filter threshold than the connectivity filter threshold.

2. Along the same lines, some of the wordings in the manuscript are quite subjective. For example, in the "offset and gain calibration" part of the Methods, the authors state "we performed some modifications to existing software to return reasonable calibration parameters" (p. 19). What are these modifications, and what is "reasonable" about the calibration parameters?

Thank you for pointing this out. We apologize for not being clear, and we have changed the wording to better express our intent here.

On pages 19-20 lines 481-484 we replaced:

"The damaged pixels caused errors in automated gain calibration (Heintzmann et al., 2018) but we performed some modifications to existing software to return reasonable calibration parameters. We were able to reliably track molecules with scalar gain, offset, and read noise variance parameters by cropping the sensor ROI so that only undamaged pixels were used in the following gain regression algorithms."

With:

"The damaged pixels caused errors in automated gain calibration (Heintzmann et al., 2018) but we were able to reliably track molecules with scalar gain, offset, and read noise variance parameters by cropping the sensor ROI so that only undamaged pixels were used in the following gain regression algorithms."

3. First line on p. 11, the authors write "softened version of the calibrated movie." What exactly does "softened" mean?

Thank you for pointing this out. We replaced the following text:

"Each frame of the resulting motion and stationary movie resembled a softened version of the calibrated movie."

With (pg 8 191-194):

"Each pixel in each frame of the resulting motion movie was temporally and spatially averaged with neighboring pixels; the resulting movie was more blurred than the calibrated movie, but maintained the signal from moving motors and background (Table 1, Figure 1C, Motion, and Video 1-4)."

And (pg 9 214-216):

"The stationary model (Table 1, Figure 1C, Motion and Stationary, and Video 1-4) used more frames than the motion model, and the resulting stationary movie is even more blurred than the motion movie."

4. More importantly, because of this issue of qualitative and subjective statements, how is a user of this algorithm to decide what parameter values to use? It would be useful if some of the spatiotemporal convolution filter parameters can be related to the expected movement of the particles for example.

Thank you for pointing this out. We addressed these issues by expanding Table 1, as mentioned above in comment #1, to include a guide on how to calculate these values for each data set, and expanded our explanation of how we measured each parameter value in the text in reference to the properties of the moving particles in our data.

5. In Fig. 1C, the LoG row: what are the white lines and pixels around the circles indicating the objects? Why are they so prominent? They are not there/barely there in the "connectivity" images.

Thank you for pointing this out. The LoG filter, or 2$^{nd}$ derivative on a gaussian blur kernel (Lindeberg, 1998), is an edge detector that detects transition points of the particle signal within an image. To better describe this, in the "LoG Filtering and Detecting Local Minima" section on page 11 lines 255-260 we changed:

"The connectivity movie was then passed through a scale space Laplacian of Gaussian (LoG) filter (Lindeberg, 1998) to enhance the edges of the signal left from the connectivity filter (Table 1 and Figure 1C, LoG). This step generated signal surrounding the moving particles, representing their boundaries (colored circles in LoG row; Figure 1C)."

To:

"The denoised KLM is then passed through a scale space Laplacian of Gaussian (LoG) filter (Lindeberg, 1998) to detect the local curvature of the signal left from the connectivity filter, using two sigma values, 1 and 1.5 pixels, representing the parameter width of the filtering kernels (Table 1, Figure 1C, LoG, and Video 1-4). This step enhanced the boundaries of the motors where there is a high transition from dark to bright signal and returned negative values at their peaks, which is why the signal appears as circles with black centers (colored circles in LoG row; Figure 1C). "

6. In the subsection entitled "tracking" on p. 13, the authors do not mention what tracking algorithm they use. Instead, they introduce it in the discussion (bottom of p. 16). This information should go up to the tracking section of the results.

As suggested by the reviewer, we made the following corrections on page 13 lines 325-327.

We changed:

> "After candidate finding, simulated motor spot coordinates were connected into trajectories."

To:

> "After candidate finding, simulated motor spot coordinates were connected into trajectories using an in-house tracking software (Relich, 2016) based on the linear assignment problem (LAP) used in u-track (Jaqaman et al., 2008)."

7. Also, what SNR was used for the tracking tests? This does not seem to be mentioned anywhere.

Thank you for the suggestion. The SNR for the simulated data ranged from 0.7 to 9. We calculated these values using the following equation described in (Salehi-Reyhani, 2017) and included this calculation in the "Methods" section on page 21 lines 519-525:

"SNR

We calculated the SNR for data of simulated motors with mean photon emissions ranging from 50 - 600 photons per full frame of acquisition based on the following equation described in (Salehi-Reyhani, 2017):

$$SNR = \frac{(S-B)}{\sigma} \quad (8)$$

Where S is the maximal peak intensity of the simulated molecules, B is the average background pixel intensity and σ is the standard deviation of the background pixel intensity."

In addition, we changed the x-axis of the Jaccard index and recall rate graphs in Figure 2 to the calculated SNR for each mean photon count.

We also updated the text in the "candidate finding" section on page 11-12 lines 277-279:

> "Fluorescent particles that represent GFP-K560 were simulated with mean photon emissions ranging from 50 - 600 photons per full frame of acquisition."

To:

> "Fluorescent particles that represent GFP-K560 were simulated with mean photon emissions ranging from 50 - 600 photons per full frame of acquisition, corresponding to a signal to noise ratio (SNR) range from 0.7 to 8.5."

8. For the tests with simulated data, i.e. Figs 2 and 3, what is the sample size? It would be good to show a mean and standard deviation per photon count in Figs 2 and 3.

Thank you for the suggestion. We ran the simulations 100 times, however each iteration of the simulation differed by only a few outlier motors drawn at the edge of the ROI, with values of 3

and 4 photons. As a result, the variation in Jaccard indices and recall rates was < 0.0045. We included the following in the Figure 2 caption on page 28 lines 695-698:

"Simulations were run 100 times and resulted in a standard deviation of < 0.0045."

9. For the sake of clarity, the authors should not interchange the terms "detection" and "tracking." For example, at the bottom of p. 12, the authors state "No algorithm tested was able to provide a tracking solution at 50 photons." What the authors really mean is detection. Of course no detection means no tracking, but there is no need to confuse the two terms.

As suggested by the reviewer, in addition to the changes we made to include the SNR, we have changed the following sentence in the "Candidate Finding" section on pg 12 lines 295-298:

"No algorithm tested was capable of providing a tracking solution at 50 photons, but Cega showed noticeable improvements at 100 photons and the median background subtracted spot finder matched performance after 200 photons, which is greater than the range of our experimental data."

To:

"No algorithm tested was capable of providing a detection solution at a SNR of ~ 0.7, but Cega showed noticeable improvements at a SNR of ~ 1.4 and the median background subtracted spot finder matched performance after the SNR exceeded 4.2, which is greater than the range of our experimental data."

10. For completeness, the authors should comment on whether/how a similar approach can be pursued if not only dim mobile objects are of interest, but also dim stationary objects. Often, the objects of interest are not mobile all the time.

Thank you for the suggestion. Cega works to segment out dim particles only when particles are less stationary than the background. Moving particles that pause can be tracked by adjusting the sliding window of the stationary movie to span the duration of the particles of interest, or else the particles of interest will be ignored. If only dim stationary particles are of interest, then a much simpler method than Cega should be used. In this case, the fluctuating noise from moving signal in the background can be removed by averaging all of the frames together and then using the LoG filter. Any moving particles will be averaged out.

To direct the user on how to adjust Cega's parameters to detect dim objects that pause, we expanded the stationary model estimation comment in Table 1 on pg 27 line 671 from:

"If tracking particles that pause for more than 31 frames, choose a larger window size. Small window sizes will eliminate stationary and paused particles in the KL-divergence model."

To:

11. Regarding the manuscript, its organization can be greatly improved. The "computational strategy" section does not cite any figures, even though there are relevant figure panels, which are then cited in the following section entitled "characterization/optimization of performance." This latter section feels rather repetitive with the computational strategy section, and as far as I can see it does not contain any characterization or optimization of performance. It is primarily a simplified, non-mathematical summary/repeat of the computational strategy section.

12. There are 8 videos associated with this manuscript, but they are not cited as far as I could see.

Reviewer #2 (Remarks to the Author):

The authors present an improved particle tracking method Cega to study single molecule movement in noisy biological systems. They have both tested the method on simulated and real data with excellent results. This is an important contribution to the community and will aid biological research in particular in studying cell biology and signaling processes. The authors have made the software available for use by other researchers.
Comments
1. The authors mention the use of EMCCD cameras, I wonder how the algorithm would perform when using sCMOS type of cameras that display aberrant hot pixel fluctuations that can affect noise statistics in a detrimental way.

"While we focus here on data obtained from EMCCD cameras, it is possible to adapt this algorithm for data acquired by other camera types including sCMOS cameras. For example, Huang et al., (2013) describe how to adapt filtering algorithms for sCMOS cameras through the use of a Poisson approximation for convolving a Poisson and Gaussian distribution."

2. Background may be structured or vary over time and not per se uniform, the authors use a temporal kernel. For Stochastic Super resolution microscopy techniques temporal (median) filters have been used efficiently and successfully. The authors did not discuss or elaborate much on this. These SSRM studies also show that structural background other give more artifacts, did the authors systematically test this for their approach?

We apologize for confusion, as well as clearly cite the correct references on pg 9 line 206. We used a temporal median filter for our background estimation, like many other SSRM groups, we added a gaussian filter to make the background comparable to our motion model. Our backgrounds are derived from real data, have structure that is found in extracted neurons, are highly non-uniform, and vary/fluctuate over time. We compared how Cega handles data from both axonal and dendritic compartments, which differ in their background, and found that Cega comparably handles both data sets.

3. Fluorophores can have very different photon counts, especially fluorescent proteins are much dimmer than organic dyes, this in combination with specific uniform or structured background levels may be critical for performance, it is not clear if this was also explored in detail, hence increasing background levels with specific photon counts per event.

Thank you for pointing this out. The ability for any detection algorithm to parse a fluorophore from a background is determined by the dominance of the fluorophore features (signal) over the background (heterogeneous and structured in all of our examples). Our backgrounds are derived from real data, have structure that is found in extracted neurons. We fixed the values of the backgrounds and we increased the values of our simulated motors to show detection efficiency. When we initially performed work on this topic, we found that increasing background fluorescence is equivalent to decreasing photon fluorescence. In other words, it's the ratio of the signal to the background that dictates our detection probability.

To address this point, we calculated the signal to noise ratio (SNR) in our simulated data and found that it covered a large range from 0.7 to 8.5, which incudes the range one would expect from dim fluorescent proteins to bright organic dyes. We changed the x-axis of the Jaccard index and recall rate graphs in Figure 2 to the calculated SNR for each mean photon count to better indicate how Cega performs as particle signal increases relative to the background.

We also included some detail for how the signal of the fluorophores in our data relate to other fluorophores in section "Candidate Finding" on page 12 lines 279-281:

"This range includes the SNR within our experimental data (Wang et al., 2014), which was measured to be ~ 4 following integration time, but also encompasses SNR expected from dimmer fluorescent proteins as well as brighter organic dyes."

4. The authors included videos, however are not clearly cited/described in the text.

As suggested by both reviewers, on pg 7 line 157, pg 8 line 194, pg 9 line 214, pg 10 lines 238 and 248, and pg 11 lines 253, 258 and 272 we have included in text references to the videos mentioned.

5. I would highly recommend to make all real and simulated (with generation scripts) data available, for example via Zenodo with a DOI.

Thank you for the suggestion. The scripts are available on GitHub https://github.com/prelich/Cega, as mentioned in the "Results" section on page 5 line 117, and the data is available on Dryad at https://doi.org/10.5061/dryad.0rxwdbrzr.

RE: Manuscript #E20-11-0744R
TITLE: "Cega: A Single Particle Segmentation Algorithm to Identify Moving Particles in a Noisy System"


Monitoring Editor (Remarks to Author):

Dear authors, Please take care of a few comments of the reviewer before submitting the final version of the manuscript.

Sincerely,

Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell


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Dear Prof. Melike Lakadamyali,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org


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Reviewer #1 (Remarks to the Author):

I would like to thank the authors for the manuscript revisions, which have made the manuscript clearer and at the same time more accessible to a reader who might want to try this new approach for detecting mobile objects in noisy images.

The authors have addressed the majority of the concerns that I raised previously.

I have a few remaining concerns:

(1) It is good that the authors now mention what algorithms they use for tracking in the Results section (p. 13). However, what is written there is not the same as what is written in the Materials and Methods "Tracking Software" on p. 22, at least not on the surface. Different publications are cited in the two places. Please reconcile and have a unified description.

(2) Thank you for listing the SNR's used for the detection tests, and for explicitly stating your SNR definition. Nevertheless, there is still no mention of what SNR was used for the **tracking** tests shown in Figure 3 and the new Supplementary Figures S1 and S2. Obviously, as detection ability depends heavily on SNR, so will the tracking ability. Please address this point and discuss it in some detail.

(3) In the new Figure S3, the authors list the photon count for the simulation. But they have now converted their other figures to SNR (e.g. Fig. 2). It would be good if they list to what SNR this photon count corresponds, for the sake of consistency.

(4) The legends of Videos 2 and 4 refer to red and green arrows, but I did not see arrows in the videos.

(5) In Videos 5-8, in the bottom 2 rows of each video, what do the magenta and cyan colors indicate?

We thank the reviewer for their suggestions, which we have now fully addressed in a point-by-point response (see below) as well as a revised manuscript. The comments are in black text and our response is in blue text.

(1) It is good that the authors now mention what algorithms they use for tracking in the Results section (p. 13). However, what is written there is not the same as what is written in the Materials and Methods "Tracking Software" on p. 22, at least not on the surface. Different publications are cited in the two places. Please reconcile and have a unified description.

Thank you for pointing this out. The tracking software we used was modified from that used in Relich 2016 and Schwartz et al., 2017, but is ultimately based on the u-track software described in Jaqaman et al., 2008.

On pg 13 lines 327-329, we changed:

"After candidate finding, simulated motor spot coordinates were connected into trajectories using an in-house tracking software (Relich, 2016) based on the linear assignment problem (LAP) used in u-track (Jaqaman et al., 2008)."

To:

"After candidate finding, simulated motor spot coordinates were connected into trajectories using an in-house tracking software (Relich, 2016; Schwartz et al., 2017) based on the linear assignment problem (LAP) used in u-track (Jaqaman et al., 2008)."

And on pg 22 lines 548-550 we changed:

"The tracking software implemented for this manuscript was adapted from the MATLAB software developed for (Schwartz et al., 2017)."

To:

"The tracking software implemented for this manuscript was adapted from the MATLAB software developed for (Relich, 2016; Schwartz et al., 2017), and is based on the software used in u-track (Jaqaman et al., 2008)."

(2) Thank you for listing the SNR's used for the detection tests, and for explicitly stating your SNR definition. Nevertheless, there is still no mention of what SNR was used for the **tracking** tests shown in Figure 3 and the new Supplementary Figures S1 and S2. Obviously, as detection ability depends heavily on SNR, so will the tracking ability. Please address this point and discuss it in some detail.

Thank you for pointing this out. On pg 13 lines 325-329 we changed :

After candidate finding, simulated motor spot coordinates were connected into trajectories using an in-house tracking software (Relich, 2016) based on the linear assignment problem (LAP) used in u-track (Jaqaman et al., 2008).

To:

Cega detection was performed on simulated data with mean photon emissions of 200, or 2.8 SNR, as this SNR is similar to that of the dimmer particles within our experimental data. After candidate finding, simulated motor spot coordinates were connected into trajectories using an in-house tracking software (Relich, 2016; Schwartz et al., 2017) based on the linear assignment problem (LAP) used in u-track (Jaqaman et al., 2008).

On pg 28 lines 703-705 and pg 30 lines 735-737 and 750-752 in the Figure 3, S1 and S2 legends we included the following to state the SNR used.

"Simulated data using axonal background signal was used where mean photon emissions were set to 200 photons, which corresponds to a SNR of 2.8."

(3) In the new Figure S3, the authors list the photon count for the simulation. But they have now converted their other figures to SNR (e.g. Fig. 2). It would be good if they list to what SNR this photon count corresponds, for the sake of consistency.

Thank you for pointing this out. 200 mean photons corresponds to a SNR of 2.8.

On pg 31 lines 766-768, we changed:

ROC plots for Cega detection on simulated data using axonal and dendritic background signal, where mean photon emissions were set to 200 photons.

To:

ROC plots for Cega detection on simulated data using axonal and dendritic background signal, where mean photon emissions were set to 200 photons, which corresponds to a SNR of 2.8.

(4) The legends of Videos 2 and 4 refer to red and green arrows, but I did not see arrows in the videos.

We apologize for this error. Videos 2 and 4 are zoomed in movies generated from Video 1 and 3, respectively, and need no other explanation other than their frame rate and scale bar.

On pg 32 lines 792 and 799 we changed:

"Green arrows indicate positions of moving particles while red arrows indicate positions of stationary particles. Movie set to play 10 fps, and scale bar set at 2 μm."

To:

"Movie set to play 10 fps, and scale bar set at 2 μm."

(5) In Videos 5-8, in the bottom 2 rows of each video, what do the magenta and cyan colors indicate?

Thank you for pointing this out. On pg 32 lines 808-810 and pg 33 lines 821-823 in the legends for Video 5 and 8, we included the following statement to connect the color choice to the direction of particle movement:

"Cyan colored tracks indicate particles moving to the anterograde (left) direction, while magenta colored tracks indicate those moving in the retrograde (right) direction."

Videos 6 and 8 are zoomed in movies generated from Video 5 and 7, respectively, and need no other explanation other than their frame rate and scale bar.

RE: Manuscript #E20-11-0744RR
TITLE: "Cega: A Single Particle Segmentation Algorithm to Identify Moving Particles in a Noisy System"

Dear Prof. Melike Lakadamyali:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell


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Dear Prof. Melike Lakadamyali:

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