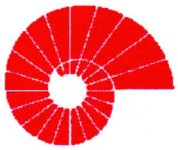

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21 January 2012

Dr. Heide Schatten
Editor, Microscopy and Microanalysis
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Ref: ID MAM-11-178: "Video-Based Tracking of Single Molecules Exhibiting Directed In-frame Motion"

Dear Dr. Schatten,

We thank the reviewers for valuable comments and appreciation of our work. We have revised the manuscript ID MAM-11-178, where necessary, according to the reviewer reports. The changes to the manuscript and the answers to the reviewers are attached to this letter. All the revisions have been made in blue color in the pdf file attached to the response letter.

We look forward to hearing from you soon regarding the revised version of the manuscript.

Sincerely Yours,

M. Yavuz Yüce
Submitting Author

Response to specific points raised by the reviewers:

We thank the reviewers for their constructive comments on our manuscript. Below, we provide the answers to the specific questions and suggestions raised by the reviewers and also indicate how the manuscript text has been modified. The page/paragraph numbers referred here apply to the revised manuscript.

Reviewer 1:

Comment 1: *"Estimating the velocity from a single image using the same approach has been published very recently. The authors should cite and briefly put in context their approach to that of: Wong, Y., Z. P. Lin, and R. J. Ober. 2011. Limit of the Accuracy of Parameter Estimation for Moving Single Molecules Imaged by Fluorescence Microscopy. Ieee Transactions on Signal Processing 59:895-911."*

Answer: As both reviewers have pointed out, the recent article

> Wong, Y., Z. P. Lin, and R. J. Ober. 2011. Limit of the Accuracy of Parameter Estimation for Moving Single Molecules Imaged by Fluorescence Microscopy. IEEE Transactions on Signal Processing 59:895-911.

is also about estimating position and velocity (among other parameters) of moving single molecules from their fluorescence images. This article can be regarded as a continuation of an earlier article by Ober *et al*.[1], which was also the starting point for our manuscript. As a result of being an extension of the same work, we naturally follow very similar steps with Wong et al. Although the main procedure in deriving the FIM is the same, the two studies are distinct in terms of the conclusions deduced from the FIM. The most prominent one of these distinct conclusions is the emphasis we make on the superior estimation performance of the mid-frame position. Other unique features of our manuscript include estimation of the molecule and background emission rates, integration of the parameter estimation algorithm into a complete tracking program, and experimental tests.

We have now added the following paragraph to the introduction section on page 3:

> "Incorporating the in-frame motion of molecules into the image formation model has been independently addressed in a very recent study through Cramer Rao lower bound calculations (Wong et al., 2011). Our work differentiates from this study mainly in recognizing the significance of the mid-frame-time position of a molecule. Using CRLB calculations and simulations, we demonstrate that the mid-frame-time position is a very convenient parameter for accurately describing the trajectory of a molecule. We subsequently exploit this observation to propose an algorithmic framework for tracking."

Comment 2: *"The application of SMT is often that of tracking a molecule that has a motion that is primarily diffusive. It should be clarified to what degree this approach can be used for that application."*

Answer: We share the reviewer's concern about diffusion, as it is a dominant form of transport on the molecular scale. Since the elementary steps in diffusion are completely random, their collective action cannot lead to a directed motion. Therefore our algorithm

[1] Ober, R. J., S. Ram, and E. S. Ward. 2004. Localization Accuracy in Single-Molecule Microscopy. Biophys. J. 86:1185-1200.

would not be suitable for tracking a molecule that solely exhibits Brownian motion. On the other hand, when the molecule also exhibits directed motion, a competing behavior arises between the effects of these two types of motions on the acquired image. While directed motion with a velocity v causes an elongation vT of the image, Brownian motion characterized by a diffusion constant D causes a statistically uniform spreading $\sqrt{4DT}$, given by the two-dimensional diffusion law. Considering the linear versus square root dependency of these two effects on T, the elongation due to directed motion will eventually dominate as T increases. For the typical values of $D = 0.1$ μm^2/s, and $v = 2$ μm/s, the critical T when directed motion starts dominating over Brownian motion turns out to be $T_c = 4D/v^2 = 100$ ms, which is a moderate value for SMT. This reasoning suggests that diffusion will not be a fundamental problem for our algorithm as long as the molecule simultaneously undergoes directed motion. Of course in this case, performance of the in-frame estimation may deteriorate, but this can be compensated by expanding the Kalman filter to include random position fluctuations of the molecule's position.

We have now added a brief comment on diffusion to paragraph-4 of the Conclusion section, and introduced a new reference[2]. We also moved the sentence "Even more general imaging models..." from the end of paragraph-2 to paragraph-4. The modified version of paragraph-4 is as follows:

"The main area of application of the presented tracking procedure lies in studying directed molecular transport, for example in living cells, fluid flows within microfluidic systems, or optical potential landscapes. In principle, stochastic Brownian motion of a molecule can be accounted for either on the level of in-frame estimation by evaluating apparent broadening of the observed point spread function (Schuster et al., 2002), or in the Kalman filter by considering the influence of the random position fluctuations of the molecule on the measured trajectory variance (Wu et al., 2010). Even more general imaging models will become possible with the advent of imaging detectors that can deliver arrival times of individual photons. Although the presented imaging model is two-dimensional, our approach can be readily generalized to three dimensions with a suitable form of the point spread function."

Comment 3: *"Check eq. 8 for a sign error. newton-raphson is x_{i+1} = x_i - f/f'"*

Answer: We thank the reviewer for pointing out this typo. We have fixed the Eq.8 in accordance to the reviewer's suggestion, and changed our referral to the equation as "Newton-Raphson".

[2] Schuster, J., F. Cichos, and C. von Borczyskowski. 2002. Diffusion Measurements by Single-Molecule Spot-Size Analysis. Journal of Physical Chemistry A 106:5403-5406.

Reviewer 2:

Comment 1: *"While the motivation for this work is obvious for a single molecule microscopist who is intimately familiar with the technical aspects of data analysis, it may not be obvious for many non-technical readers. Therefore, the authors need to address the relevance of this work in the introduction. A comment about why single molecule movement should be taken into account for its localization would be helpful."*

Answer: In accordance with the reviewer's suggestion, we added the sentence "Since this extended model ..." to the first paragraph of page-3, and modified the rest of the paragraph as underlined below:

> "In this article we apply the same approach to molecules in motion. To the best of our knowledge, all the present tracking algorithms ignore molecules' motion within one-frame time, and fit the data with the point spread function of a stationary emitter. We follow an alternative method, and use a new imaging model that also takes into account the motion of the molecule during the exposure time of a frame. Since this extended model better represents the real imaging process, it is expected to yield more accurate position estimates for a moving molecule. As inherently including motion, the model also allows molecules' velocity to be estimated from single frame data."

Comment 2: *"The authors need to cite prior work related to the manuscript. Recently, Wong et al (IEEE Transactions in Signal Processing, 2011, 59:895-911) have carried out a rigorous analysis of the Fisher information matrix for a moving single molecule. Other groups have also considered the effect of single molecule motion in the quantitative analysis of the data (e.g., Voisnne et al, Biophysical Journal, 2010, 98:596-605)."*

Answer: The article by Wong *et al.* was also mentioned in the first reviewer's comments. We kindly refer the second reviewer to our answer above. The work by Voisinne *et al.* uses a Bayesian method for the analysis of recorded molecular trajectories that are assumed to be accurately known. Our algorithm on the other hand is developed to find an unknown trajectory of a mobile molecule, which can be further analyzed by the methods developed by Voisinne *et al.*

Comment 3: *"In Section 2.4.1, the authors describe the estimation framework. From the definition of their parameter vector Θ, it appears that the authors assume the width of the image profile to be known (σ if the profile is a Gaussian). However, in most practical situations, the width is also estimated. How are the authors fixing the value of sigma in their experimental data?"*

Answer: We have determined the σ parameter from the images of stationary dye molecules in two ways: i) by fitting a 1-D Gaussian function to intensity profiles, and ii) by implementing a maximum likelihood algorithm to estimate the σ parameter. Both methods were in close agreement. In the subsequent tracking experiments, we assumed that the value of σ remained constant.

To clarify our procedure we modified the corresponding part on page-5 as:

> "The point spread function of the setup was determined from stationary single molecule images, and corresponded to $\sigma = 1.2$ pixel $\approx$ 108nm, when approximated with a Gaussian function. This σ value has been used in analyzing recorded movies."

Since we have considered 2-D single molecule tracking, we have confined ourselves with a constant σ. Our algorithm can be extended easily by including σ into the parameter space for general 3-D single particle tracking.

Comment 4: *"In eq. 8 the authors use L to denote the number of iterations. However, L is also being used to denote the likelihood function. The authors need to fix this duplication of symbols."*

Answer: We thank the reviewer for noticing this duplication error. We replaced the *L* in Eq. 8 with the symbol Ω.

Comment 5: *"The rank deficiency of the Fisher information matrix is a very interesting observation and it is also somewhat intriguing. For the stationary case, it is clear that two of the rows of the Fisher information matrix would be linearly dependent. Therefore, the matrix will not be invertible. As for the reparameterization in terms of the mid frame coordinates $x_c = x_0 + vt/2$, the authors need to be more specific as to how they parameterize the model in terms of x_c. Are they recalculating the Fisher information matrix for x_c? If so, what is the parameter vector?"*

Answer: As correctly pointed out by the reviewer, the rank deficiency of the Fisher Information matrix and the accuracy result regarding the midframe point $\begin{bmatrix} x_c \\ y_c \end{bmatrix}$ can suggest midframe based parametrization for the linear motion. However, we decided to keep our initial-point-based parametrization in the article, for both performance analysis and the algorithm, in order to attract more attention to this interesting result. As far as the algorithm is concerned, we calculate the estimates of the mid frame position parameters $\begin{bmatrix} x_c \\ y_c \end{bmatrix}$ from the estimate of the parameter vector Θ through

$$\begin{bmatrix} x_c \\ y_c \end{bmatrix} = \underbrace{\begin{bmatrix} 1 & 0 & T/2 & 0 & 0 & 0 \\ 0 & 1 & 0 & T/2 & 0 & 0 \end{bmatrix}}_{\mathbf{H}} \widehat{\boldsymbol{\Theta}}.$$

Regarding the performance analysis based on CRLB, the singularity of the Fisher Information Matrix implies unresolvable ambiguity in unbiased estimation of some linear combinations of the parameter vector for the weight vectors lying in the null space directions of the Fisher Information Matrix. Following the discussion in the article the weight vectors corresponding to the midframe locations (i.e. the rows of **H** matrix defined above) do not lie in this space.

To clarify this point, we modified the corresponding text on page-14 as follows:

"In fact, the whole parametrization in this study could have been done in the form

$$\begin{bmatrix} x(t) \\ y(t) \end{bmatrix} = \begin{bmatrix} x_c \\ y_c \end{bmatrix} + \begin{bmatrix} v_x \\ v_y \end{bmatrix} (t - T/2) \quad t \in [0, T],$$

however we decided to keep our initial point based parametrization in order to attract more attention to this interesting result."

Comment 6: *"The results and discussion section needs re-organization. The main concern I have with this section is that it is very long and the reader is given no preamble of what to expect. This makes the reading of this section very difficult. The authors should break the results into different subsections. With appropriate headings, the reader, when glancing through the paper, will at least know what is being discussed in these pages."*

Answer: In accordance with the reviewer's suggestion, we break the "Results and Discussion" section into two subsections, entitled "Low-level analysis" and "High-level analysis". Our changes regarding re-organization are as follows:

- We replace the first sentence of the "Results and Discussion" section with the following introductory information:
  "In this section we evaluate the performance of our tracking algorithm at the low- and high-levels described in the Algorithm section. In testing the low level analysis (in-frame estimation), we first use CRLB calculations to find the smallest achievable error in estimating parameters of the proposed imaging model under typical experimental conditions. We then apply our estimation algorithm to simulated images of a moving molecule, and compare its success with the theoretical outcomes of the CRLB calculations. For testing the high-level analysis (tracking), we use actual experimental data recorded as described in the Materials and Methods section. We compare the molecule trajectories obtained from analysis of the movies with the ones obtained from the piezo-stage sensor."

- This introduction to the section is followed by the "Low-level analysis" subsection starting from the sentence:
  "Starting with the in-frame estimation, we first consider the case where ..."

- The discussion on bias-variance trade-off has been moved to the end of the "Low-level analysis" subsection as a separate paragraph.

- The "High-level analysis" subsection starts from the paragraph:
  "So far, the discussions have focused on the performance of the in-frame ..."
  on page-16.

- Separate paragraph introduced starting from the sentence:
  "In these experiments, we compare the measured ($\mathbf{y}_k$) and filtered ($\hat{\mathbf{x}}_{k|k}$) ..."
  on page-16.

- New sentence in front of the last paragraph of page-17:
  "In addition to the linear motion case presented above, we also wanted to test our algorithm for a more general type of motion."

Comment 7: *"Why was the velocity chosen to be 3.11 microns/s (7 pixels/frame)? Was there any specific for this choice or was it arbitrary?"*

Answer: The choice of 7 pixels/frame was not for any specific reason other than conveniently demonstrating the effects (finite window size, and ill-conditionedness of the FIM) that we discuss on Page-13, regarding Figure 2A. Under the imaging conditions used, 7 pixels/frame corresponded to 3.11 μm/s.

Comment 8: *"The authors need to standardize their units to either microns, microns/s, s, etc., or pixels, pixels/frame. At various places in the manuscript, the authors switch back and forth between the two units and this is not very helpful. Representation of distances and speeds in the single molecule literature is mainly in terms of microns/nanometers and microns/s or nm/s, respectively."*

Answer: In the manuscript, we mostly use pixel or pixel/frame, and give the corresponding value in nm,μm or nm/s,μm/s within parenthesis. In order to maintain consistency, we brought all the units to this format, except for the values we use in the process noise covariance ma-

trix, Q. Since Q has no connection with the imaging conditions, we thought it would be confusing to specify its elements in pixel^2 or (pixel/frame)^2. The changes to the manuscript regarding units include:

- Page-15, second paragraph: "…The maximum error within the studied velocity range turns out to be < 1.5 pixel/frame (< 2.67 μm/s) for v_x, and < 0.5 pixel (< 44.4 nm) for x_c. The growing behavior ..."

- Page-17, first paragraph: "We see that both measured and filtered data reveal the actual motion within average errors of 0.17 pixel (15.54 nm) and 0.11 pixel (9.65 nm) respectively."

- Page-17, first paragraph: "Since the sensor reading itself has a standard deviation of 0.03 pixel (2.6 nm) in the (stationary) y-axis (see Figure 3 A), error in the filtered data cannot be smaller than $\sqrt{2} \times 0.03 = 0.04$ pixel (3.6 nm)."

- Page-17, last paragraph: "Despite the absence of corrections from Kalman filtering, we see in Figure 4 B that the error in position estimation still remains smaller than 1 pixel (90 nm), and has an average value of 0.42 pixel (37.43 nm)."

- Page-18, second paragraph: "We were able to track molecules under uniform motion with an average position error of 0.11 pixel (9.65 nm)."

Comment 9: *"The figure numbers referred in the text do not match the actual numbering of figures. For instance, in the results section the authors refer to Figure 1A, 1B and 1C, which should be figure 2A, 2B, 2C since Figure 1 does not even have different panels and only contains a schematic of the image formation model. Similarly, other figure numbers in the text need to be fixed."*

Answer: Due to a problem with the Latex file, Figure 1 was mistakenly assigned as Figure 6, and the remaining figures' numbers were all assigned to those of the previous ones. This problem is now fixed.

Comment 10: *"The use of the term "CRLB for the standard deviation" in figure 2A and 3A is misleading. By definition, CRLB refers to the lower bound on the variance of the parameter estimates. The authors could either denote the y label as square root of CRLB which is cumbersome, or refer to the y-axis as the limit of the accuracy of estimates."*

Answer: We would like to thank the reviewer for this suggestion. We modified the y-axis labels of Figures 2A and 3A as "square root of CRLB".

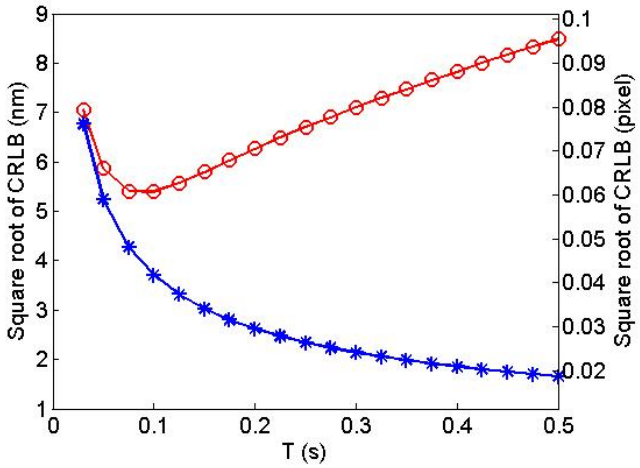
Comment 11: ***"****In Figure 2, the authors have plotted the limit of the accuracy of the estimates for x_0 and x_c. It is very important that the authors also plot the limit of the accuracy of x_0 for the stationary case. This goes back to my earlier comment about elucidating the importance of this work. The limit of the accuracy for the stationary single molecule would provide a very important benchmark to compare and appreciate the benefit of considering the motion of single molecules. For instance, the reader can then immediately determine for which velocities there is significant deviation between the moving version and the stationary version of the limits."*

Answer: The primary motivation of this study is to improve position estimation in single molecule tracking. In order to achieve this, we use an imaging model that is particularly developed for a moving molecule. In single molecule tracking, due to the very purpose of the technique, one will always be dealing with moving molecules. Therefore we found it more

convenient to provide a comparison between alternative models, and estimated positions of moving molecules from the same set of simulated images by i) using the proposed model, and ii) using a modified version of the proposed model that neglects in-frame motion. This provided us the opportunity to see the effect of including versus neglecting the in frame motion, as shown in Figures 2B, 2C, 3B, and 3C. In these figures, the red circles show bias (Figures 2B and 3B) and standard deviation (Figures 2C and 3C) in position estimates obtained using the proposed model (x_c), and similarly black diamonds show bias (Figures 2B and 3B) and standard deviation (Figures 2C and 3C) in position estimates obtained using the modified version of the proposed model that neglects in-frame motion (x_m).

The accuracy in estimating the position of a stationary molecule is not considered in the manuscript, but we provide this information here as a brief discussion. The following figure shows as a function of T, the square root of CRLB in estimating position of a stationary molecule using the modified version of the proposed model that neglects in-frame motion (asterixes). Molecule and background emission rates, λ_0 and λ_{bg} were set to 15000 and 300 photons/s, and a window size of 30×30 pixels was used. For comparison purposes we also plot the square root of CRLB in estimating mid-frame position of a moving molecule using the proposed model (circles; same speed and λ_0, λ_{bg} rates as in Figure 2 A of the manuscript). The difference between these two curves is a result of the motion. When the molecule is stationary, increasing T increases the number of detected photons, and improves estimation accuracy as expected. However when the molecule is moving, increasing T also causes more distortion of the image, and while the total number of detected photons still increases, number of detected photons per pixel remains constant (within the shot noise) for the uniform motion case considered here. This dependency suggests an optimum [speed, exposure time] pair that provides the best estimation performance. In the initial stages of our study, we obtained ≈ 3.5 pixel/frame for this optimum [speed, exposure time] pair, but didn't continue investigating it further, for we couldn't see a practical use of it. In a real experiment, molecules usually possess a distribution of velocities, and the exposure time that is optimum for a particular molecule may not be so for another molecule having a different velocity. It may also be the case that a molecule moves with different velocities throughout its trajectory. Moreover, the slight dependency of the accuracy limit in x_c estimation on T over a wide range of T suggests that the choice of T in a real experiment is likely to be determined by other factors as described on Page-15 first paragraph of the text.

Consequently, although a comparison between the fundamental limits of the localization accuracies of stationary and moving molecules provides intuitive results, we preferred to limit the content of the manuscript to moving molecules.



Comment 12: *"In page 13, last paragraph, first sentence, the authors state that the standard deviation of their estimated parameters and the coresponding CRLB are "very close". However, upon examining the figure, the two number are close only for a certain range of values. In particular, the*

standard deviation and the CRLB are widely different for small ($t < 0.1$ s) and large ($t > 0.4$ s) exposure times. The authors either need to explain this discrepancy at this point or at least allude to the explanation that they provide later in the section."

Answer: In accordance with the reviewer's suggestion we now specify the range of T values as 0.1 s < T < 0.4 s, for which the standard deviation in estimates of x_0, v_x, and x_c obtained from simulated images are in agreement with their corresponding CRLB limits. We have also added a sentence about the coming discussion on bias-variance trade off. This paragraph has now become as follows:

> "Similarly, Figure 2 *C* compares the standard deviation in estimation of x_0, v_x and x_c (interpreted as the precision) with the CRLB limits of Figure 2 *A*, and shows that the two are very close for T values from 0.1s to 0.4 s. The window effect … assumption of the CRLB analysis. Further discussion on the discrepancy at the short T end is going to be provided following the next figure, where a more direct connection can be made with the Algorithm section. Estimation of x_c …"

Comment 13: *"In page 14, last paragraph, 8th line there is a typo - "groving" behavior."*

Answer: The typo has been corrected.

Comment 14: *"In Figures 2B, 2C, 3B and 3C, the authors need to explain what the diamond line denotes, as the figure legends does not provide any information about this."*

Answer: The diamonds in these figures denote the bias (for Figures 2B and 3B) and standard deviation (for Figures 2C and 3C) in molecule's position estimation, when a modified version of the proposed imaging model that neglects in-frame motion is used. This demonstrates the advantage of the proposed model with in-frame motion over standard models assuming the molecule to be stationary within the acquisition time. The position estimates obtained when neglecting the in-frame motion are denoted by x_m, as described on page-13 (last paragraph, last sentence) of the manuscript. We have modified the captions of Figure 2 and 3 for clarity, and also corrected the typo "cicles". The new captions are as follows:

"Figure 2: Performance of the proposed estimation algorithm as a function of T for parameters v_x (green triangles), x_0 (blue squares), and x_c (red circles). Tracking performance is evaluated on the basis of (A) CRLB limits, (B,C) bias and standard deviation of estimates from simulated images. For comparison, corresponding characteristics of x_m obtained from same set of simulated images are also shown (black diamonds). For all presented data, v_x is kept fixed at 3.11 μm/s (7 pixel/frame at T = 0.2 s)."

"Figure 3: Performance of the proposed estimation algorithm as a function of v_x for parameters v_x (green triangles), and x_c (red circles), based on their (A) CRLB limits, (B,C) bias and standard deviation of estimates from simulated images. For comparison, corresponding characteristics of x_m obtained from same set of simulated images are also shown (black diamonds). T is kept fixed at 50 ms for all presented data."

Comment 15: *"In the appendix, page 1, First order derivatives section, the first line has a typo. The word "genearal" is incorrectly spelt. The same typo also shows up in page 5 of the appendix, first line under the Second order derivatives section."*

Answer: These typos have been corrected.

Comment 16: *"In eq. 3, the authors define $p_k(t)$ to be a double integral over A_k, where A_k denotes the k^{th} pixel. However, in the appendix, the authors represent the derivative of $p_k(t)$ wrt x_0 as a single integral along the y-direction of the pixel. How did the authors arrive at this equation? Perhaps explicitly specifying the intermediate steps (and assumptions if any) will be useful."*

Answer: We would like to thank the reviewer for this comment aiming at clarifying our article. We changed the related part of the supplementary section as shown below:

"We can write $p_k(\tau)$ as

$$p_k(\tau) = \int_{y_{k1}}^{y_{k2}} \int_{x_{k1}}^{x_{k2}} g(x - x_0 - v_x\tau, y - y_0 - v_y\tau)dxdy$$

$$= \int_{y_{k1}-y_0-v_y\tau}^{y_{k2}-y_0-v_y\tau} \int_{x_{k1}-x_0-v_x\tau}^{x_{k2}-x_0-v_x\tau} g(x,y)dxdy$$

Therefore, the partial derivative of $p_k(\tau)$ with respect to x_0 can be written as

$$\frac{\partial p_k(\tau)}{\partial x_0} = \int_{y_{k1}-y_0-v_y\tau}^{y_{k2}-y_0-v_y\tau} \frac{\partial}{\partial x_0}\left(\int_{x_{k1}-x_0-v_x\tau}^{x_{k2}-x_0-v_x\tau} g(x,y)dxdy\right)$$

$$= \int_{y_{k1}-y_0-v_y\tau}^{y_{k2}-y_0-v_y\tau} (g(x_{k1} - x_0 - v_x\tau, y) - g(x_{k2} - x_0 - v_x\tau, y))\, dy.$$

"

Other changes in the manuscript:

Although the “Algorithm” part was originally written as a separate section, due to a problem with our Latex file, it appeared under the “Materials and Methods” section. This mistake has also been corrected.