

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors present a method for assessing the asymmetry of how the cell partitions objects upon cell division. To the best of my knowledge, the method is novel and can be of interest for future research. Overall, the work appears solid, though in some instances (see below) I think more evidence is warranted.

As a point of clarity, I feel the authors should stress that the pivotal aspect of their method is the fact that they sort the cells. The rest essentially follows from this approach.

Related to that, I think it would be worthwhile to discuss the effect of (artificially) choosing a narrow initial population. Obviously, the authors are thereby biasing the results to those particular cells, which may introduce artifacts or, in any case, have an effect.

The choice of Gaussian for fitting procedure (Eq. 2) should be discussed and better motivated. The data (Fig. 2d), while not representing the sorted population, nevertheless looks more log-normal than Gaussian. Related to this, it would seem reasonable to actually show the fits (in one dimension, rather than the 2D versions shown in Fig. 2b, from which I can tell very little about the goodness of fit).

The authors say in the Abstract that the results are “incompatible with an independent division mechanism”, yet their model assumes a binomial partitioning which is based upon independent partitioning. Some clarification on this would be useful.

The authors do not seem to take into account the fact that (at least) the mitochondria are individual objects and the binomial partitioning should be applied to the organelle, not its fluorescence intensity. A similar point may be made for the cytosol and lipids, though I think it is less important for them.

p. 10 penultimate paragraph: I understand the utility of having several markers, where one (the symmetrically partitioning one) helps identifying the generations. However, I do not understand how the authors take into account (or why they do not need to take into account) the loss of fluorescence of the other dye(s) in their model.

It would be useful if the authors discuss the influence of different cell cycle lengths among the cells, as well as a potential slowing down of the cycling that may very well be present in their experiments (since they consider up to 5 generations). From my understanding of the work, it should not pose too much of an issue (as long as the generations do not start overlapping) but it would be worthwhile to discuss it.

Minor comments:

The title does not reflect the work in my opinion.

I think the implication that asymmetric partitioning is responsible for phenotypic variability in cancer cell lines should be removed. As far as I can tell, this is only discussed in the Abstract so obviously no

real discussion is provided. If the authors do want to make this case, then I think it needs to be discussed at much more length, because it is not an immediate outcome of the work.

Fig. 4e-f. This appears to be, at best, preliminary, with a lack of reproducibility between experiments.

Part of the Discussion seems misplaced and is indistinguishable from information covered in the Introduction. This could be consolidated.

The language is in need of a bit of a spell-check before final publication, but it is nothing that prevents understanding of the work.

Reviewer #2 (Remarks to the Author):

In their manuscript, Peruzzi et al. develop a FACS-based method to analyze the asymmetry in the distribution of fluorescently-labelled organelles in a proliferating population of human Jurkat T cells. The authors further demonstrate that experimental results obtained on differential organelle distribution can be explained by assuming a minimal model of asymmetric partitioning where the components are divided according to biased binomial statistics. For the most part, the experiments described in this manuscript are well designed and properly executed, and the figures are clearly presented. However, and although interesting, the study falls short in terms of the biological relevance of its conclusions and in representing a significant advance in our present knowledge in the field. Prior analyses already showed that several molecules and organelles are differentially distributed during asymmetric divisions in cancer cells. As such, asymmetric cell division and differential centrosome inheritance have been demonstrated to occur in human neuroblastoma cells (Izumi and Kaneko, 2012). Similarly, the Numb polarity factor seems to control the segregation of subcellular vesicles during the division of colorectal cancer stem cells (Hwang and Yang, 2016). These and other references, in fact, should have been included in the introduction, which somewhat excessively relies on general reviews. Finally, and despite the authors incorporate an asymmetric binomial partitioning model to adjust the experimental data that provides an interesting novel methodological contribution to the analysis of differential organelle distribution, the use of a FACS-based approach to sort out different populations of cells and evaluate organelle partitioning has also been widely used before. Besides these general considerations, other main issues regarding the manuscript are:

1.- The authors analyze the partitioning of cytoplasm, cell membrane and mitochondria during the proliferation of Jurkat lymphocytes derived from human T cell leukemia. Their results suggest that while in Jurkat cells the cytoplasm is evenly partitioned between the daughter cells, asymmetric distribution of membranes and mitochondria can be observed. However, a main objective of the manuscript was to help elucidating whether variability associated to the asymmetric partitioning of cellular components could play an important role in tumorigenesis. The authors could have thus exploited the designed experimental and computational protocol to dig deeper into this matter. In this way, by using their methodology to follow the distribution of organelles in populations of cells during mitosis in a relatively fast and easy manner, the authors could have evaluated whether there exist differences in the partitioning of cytoplasm, cell membrane and mitochondria during the proliferation of Jurkat T cells and in populations of immortalized non-transformed T cells, such as TERT-expressing T cells. Since TERT-expressing T cells do not develop genetic changes characteristic of malignant transformation and maintain intact the mechanisms that regulate growth (Barsov, 2011), this comparison between transformed and non-transformed cells could thus reveal

differences in asymmetric partitioning of organelles that might shed light on whether their differential distribution had a possible role of in tumor evolution.

2.- The dyes used to mark the cell membrane, cytoplasm and mitochondria have different fluorescent properties and photostabilities. This is a factor that could definitely have an impact on the outcome of the FACS analyses. Since cell markers coupled to different fluorophores are commercially available, the authors could have easily performed the same experiment with a different combination of dyes (e.g., CellTrace™ Yellow, MitoTracker™ Red FM and PKH67 green fluorescent cell linker kit for general cell membrane labeling) as a control for the methodology.

3.- Peruzzi et al. speculate that asymmetric cell membrane partitioning may be linked to aggregated of membrane proteins formed after T cell activation. In order to verify this hypothesis, the authors acquired confocal microscopy images that they indicate that showed aggregation clusters at the membrane level for the PKH fluorescence in many cells (page 10, line 217). However, these images are not shown. The authors should either include the confocal images or remove this statement. Likewise, when discussing the results from Figures 2c-d, it is indicated that the same behavior is observed when the mitochondrial marker is used instead of the PKH maker, but the data is not shown (page 6, line 126). These results should be included as supplementary data.

Finally, some minor concerns are:

- 1.- The text should be reviewed for proper syntax and word choice. Results should be written in past tense. Finally, there are also several typos (e.g., “cellular component” instead of “cellular components” (page1, line 2), “PHK” instead of “PKH” (page 3, line 36), “fitt” instead of “fit” (page 4, Figure 2 legend), etc.).
- 2.- The definition of asymmetric cell division in the introduction is not accurate. In general, an asymmetric cell division results in two cells that differ in size, cellular components and/or fate, but does not necessarily ends up with one of the cells differentiating into a specific cellular type.
- 3.- The figure legends are, in many cases, missing important information. In this way, while in Figure 2 it is indicated what do the dark blue, red and green dots represent, no information is provided about the light blue and yellow dots. Likewise, it should be clearly stated what do the star, triangles and circle represent in Figure 4. These symbols are referred as “dots”, which leads the reader to confusion. Finally, in the legend for Figure 4, the statement “the results are very consistent” could be removed. This is a consideration that, if necessary, could be introduced in the text.
- 4.- In Figure 2b, percentages of cells in each generation are reported in an insert as bar plots. However, no scale for the plots is provided.

Dear Reviewers,

We would like to thank the Reviewers for their thoughtful comments and suggestions. Changes made in response to the points raised are reported in **red** if the text was removed and in **blue** if the text was added in the 'Peruzzi_Main_Diff_CommsPhys.pdf' file. Our replies to the Reviewers' comments are given in the following in **blue**.

Thank you for your work.

Kind regards,

Giorgio Gosti

for Giovanna Peruzzi, Mattia Miotto, Roberta Maggio and Giancarlo Ruocco

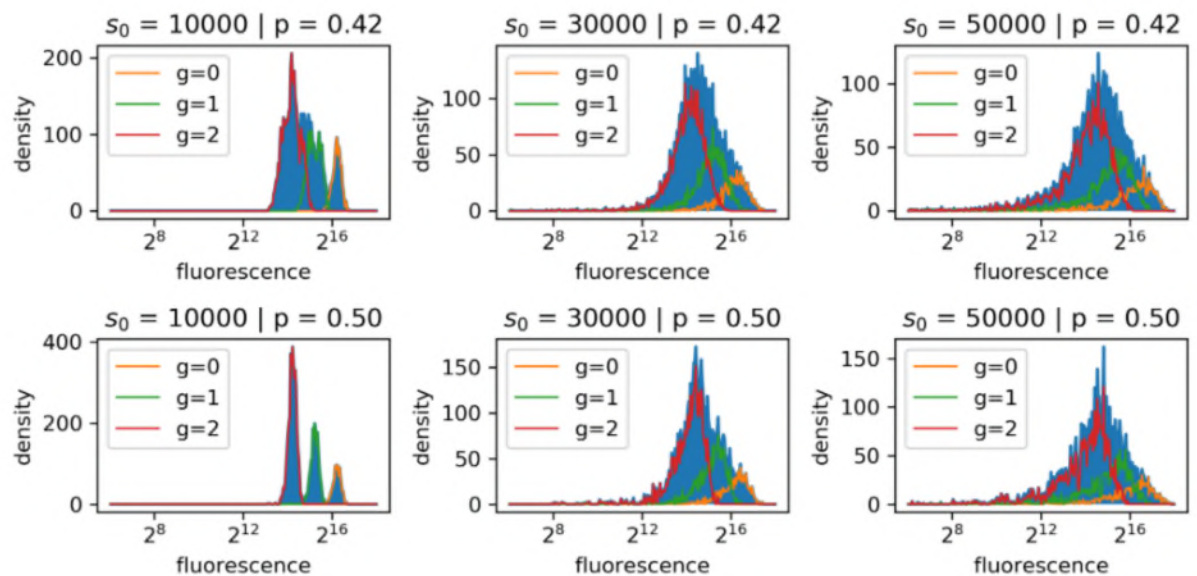
Reviewer #1

The authors present a method for assessing the asymmetry of how the cell partitions objects upon cell division. To the best of my knowledge, the method is novel and can be of interest for future research. Overall, the work appears solid, though in some instances (see below) I think more evidence is warranted.

We thank the Reviewer for the positive assessment of our work. We address all the points raised in detail below.

As a point of clarity, I feel the authors should stress that the pivotal aspect of their method is the fact that they sort the cells. The rest essentially follows from this approach.

The Reviewer is absolutely right on the importance of the sorting procedure. Indeed, sorting the cells after the staining allows us to select a narrow initial distribution which is essential to distinguish the subsequent generation peaks, which in certain circumstances tend to widen gradually. This can easily be seen from the binomial model upon starting the replication dynamics from initial distributions with different variances.



The plots above show the fluorescence distribution for two fluorescences with different asymmetry parameters p . The plots in the first row show the fluorescence distribution for a fluorescence marker of an organelle that asymmetrically partitions with $p = 0.42$. Each plot presents three peaks, orange is the initial sorted population, and the green and red peaks are respectively the following generation 1 and 2. The blue distribution is the result of the three peaks merging. Each of the three plots shows a different initial condition characterized by a variance s_0 . The plots of the second row consider a symmetrically segregating fluorescent organelle $p = 0.5$. In general, the higher the initial variance (s_0) the harder to distinguish the generations (from gen 0 to gen 2). The reason is that when the variance becomes too broad the peaks

merge into a single peak. This effect becomes more and more important as the asymmetry of the partitioning increases ($p < 0.5$) since the resulting subpopulations tend to merge together.

Furthermore, sorting also enables us to start with an initial distribution that is distant from the background fluorescence. As the initial distribution is set further away from the background fluorescence, we are able to follow the proliferation for a longer time, thus we can distinguish more generations. It is worth noticing that this aspect is also dependent on the dyes used which have specific background fluorescence levels.

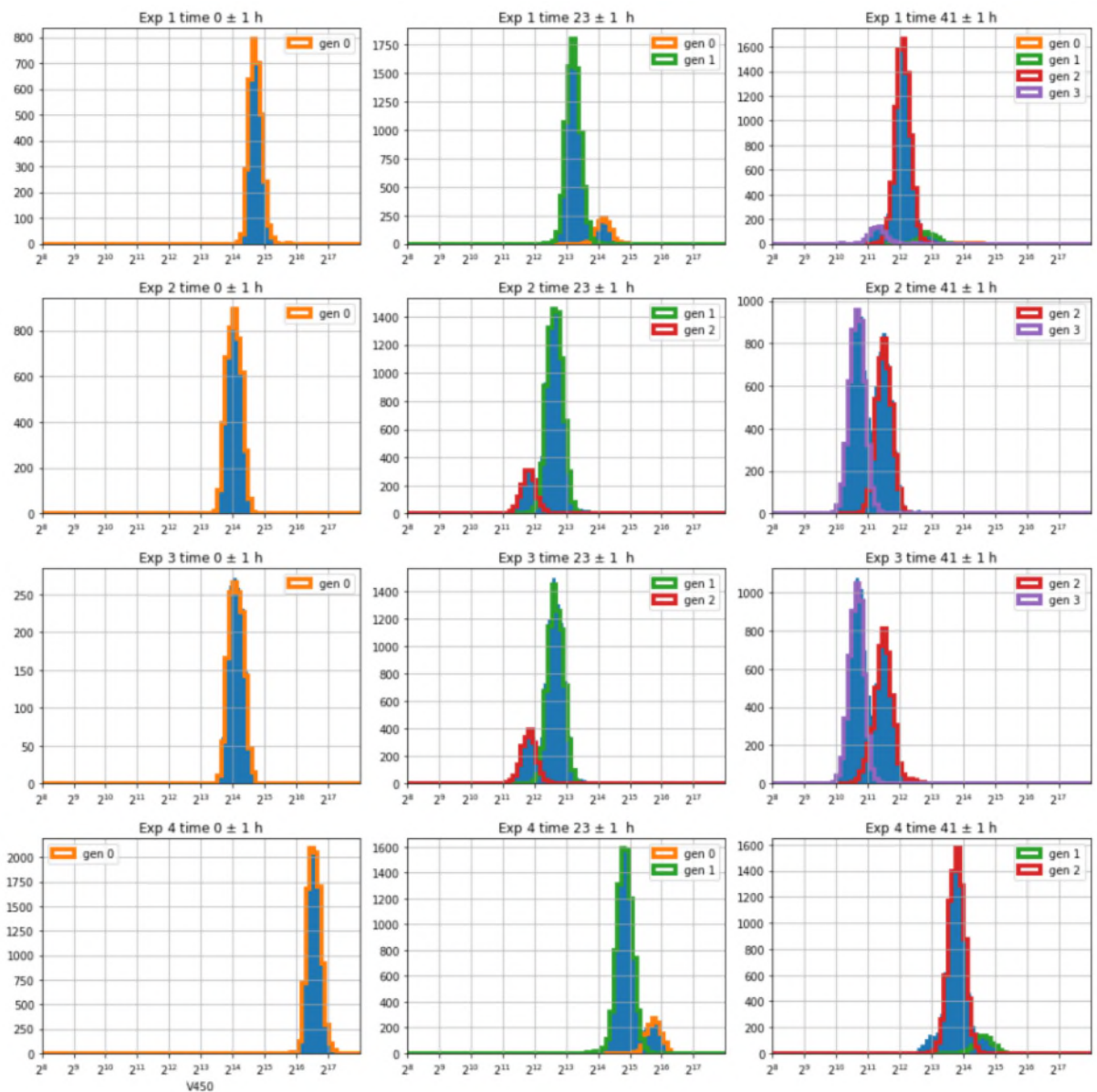
In addition to these technical aspects, there is a third benefit of the sorting procedure that has an important biological implication, i.e. to synchronize the population. As the Reviewer raised this issue in the following point, we continue the discussion there. We have now added a discussion on these aspects (Sec. II in SI) in the novel version of the manuscript and added the above plots to the SI (Fig. S1 in SI).

Related to that, I think it would be worthwhile to discuss the effect of (artificially) choosing a narrow initial population. Obviously, the authors are thereby biasing the results to those particular cells, which may introduce artifacts or, in any case, have an effect.

Indeed, because we sort the cells with narrow gates, centered on the bulk of the fluorescence signal distribution for each dye, we obtain pools of cells that carry similar morphological characteristics. More specifically, especially sorting for cell trace markers which are cytoplasm markers, we sort cells with a certain size. But the issue of sorting cells that carry similar morphological characteristics is more complex. We must take into consideration that a proliferating cell population is a dynamical system, consequently cells in this population go through a sequence of phases characterized by specific morphologies. Thus when we sort a cell population we have one of two situations: either we start with a morphologically homogeneous population of cells and consequently when we sort we tend to synchronize this population in a specific phase, or we start with an initially heterogeneous cell population, and, when we sort, we risk selecting a particular more or less synchronized subset of cells. In our case, Jurkat cells are known to be a very homogeneous cell line especially under the specific condition set in the protocol. To further reinforce these considerations in the rest of this response we discuss two points. First, we discuss the advantages of synchronizing an homogeneous population with cell sorting. Second, we show that in our experiments we used sorted subsets of cells in different phases, and that from these different subsets of cells we obtained the same values of asymmetric partitioning (as quantified by the p parameter). Thus we can argue that these initial conditions do not bias our result by artificially sorting a non representative subset of the population.

As argued in [1], synchronizing by sorting is an important starting point, because it allows us to follow a specific cell population subset similar per size

and internal complexity despite fluorescence intensities, and allows us to better track changes in the cell population growth. Furthermore, synchronizing cells with sorting methods has fewer consequences compared to whole-cultured cells [1] because it does not require artificially blocking the cell cycle in abnormal quiescent states that are not characteristic of the natural dynamics of the freely growing cell population. The fact that by sorting our initial cell population we obtained cell samples that are synchronized on different cell phases is shown in our experiments' data. Because we have collected samples for each experiment at different time points, which show how different experiments presented dynamics of the evolution of the cell generation peaks that were in two different major phases.



In the plots above, we show different time points of the acquisition of CTV fluorescence intensity (V450). The rows correspond to Experiments 1, 2, 3, and 4. The column corresponds to three different time points measured from the

initial sorting: 0 hours, 23 hours, 41 hours. We see that Exp 1 and Exp 4 are synchronized on the same phase, and Exp 2 and Exp 3 are synchronized on another phase. We can see this from the plots because, in Exp 1 and Exp 4, at time 23 hours the peak corresponding to generation 0 is still present and the peak corresponding to generation 2 has not emerged. On the contrary, in Exp 2 and Exp 3, the generation 0 peak is not present, but peak 2 is present. We see a similar trend at the 41 hour time point with Exp 1 and Exp 4 being slightly behind Exp 2 and Exp 3, with generation 3 not developed, and still presenting a small proliferating generation 1 peak.

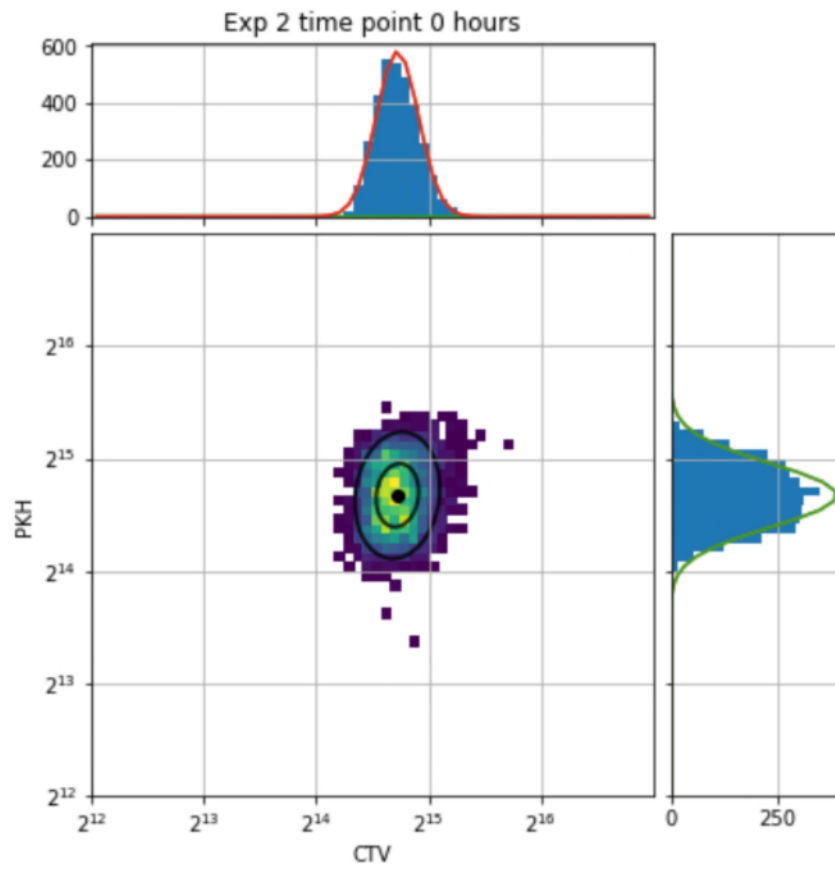
The fact that at each experiment the sorting procedure synchronized cells at different cell cycle phases and that we obtained the same p value for different subsets of the cell population, shows us that we are not biasing the results to those particular sorted cells. We have now added a discussion on these aspects in the novel version of the manuscript and added the above plots to the SI (Sec II and Fig. S2 in SI).

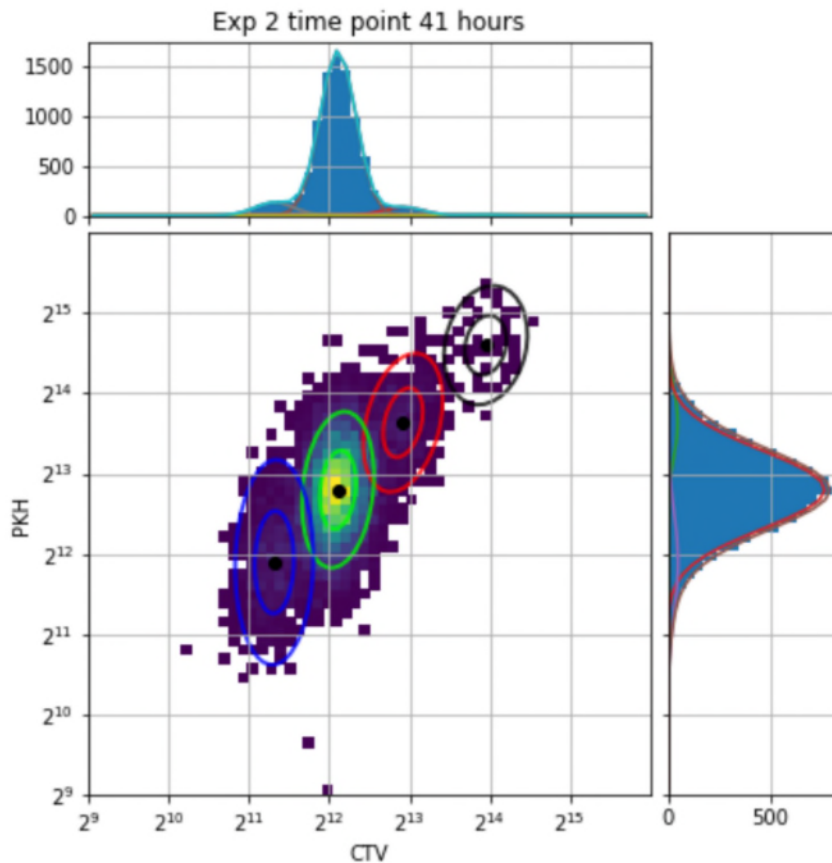
- [1] Cooper, S. (2019). The synchronization manifesto: a critique of whole-culture synchronization. *The FEBS Journal*, 286(23), 4650–4656. <https://doi.org/10.1111/febs.15050>

The choice of Gaussian for fitting procedure (Eq. 2) should be discussed and better motivated. The data (Fig. 2d), while not representing the sorted population, nevertheless looks more log-normal than Gaussian. Related to this, it would seem reasonable to actually show the fits (in one dimension, rather than the 2D versions shown in Fig. 2b, from which I can tell very little about the goodness of fit).

It is important to point out that we use the Gaussian Mixture Model (GMM) to detect the peaks in the data, and we used the GMM on the log of the fluorescence data points. We do not use the gaussian mixture model to directly estimate our parameters. We noticed that when the fluorescence intensities are plotted on a log scale their distributions are distributed approximately as a Gaussian. The Figures below show the 2D density distribution obtained for the Exp 1 at the 0 hour time point and at the 41 hour time point. On the sides of each 2D scatter plot, we presented the marginal 1D distributions for the CTV and the PKH. As the Reviewer mentioned, the marginal distributions clearly show how the Gaussian approximately fits the distribution. As it can be seen, the gaussian distributions give a good recount of the observed peaks plotted in log scale. If we compare the observed density distribution and the derived optimal mixture of Gaussian distribution we can compute the residual sum of squares. We get respectively for the first figure that corresponds to the time point 0 hours the residual sum of squares for the normalized distribution $\Sigma = 6.4 \times 10^{-4}$, and for the second figure that corresponds to the 41 hour time point $\Sigma = 1.12 \times 10^{-4}$. To understand the actual scale of the residual sum of squares,

the reader must consider that we computed it on the normalized distribution, thus in the worst scenario, the sum of squares for non overlapping distributions should be 1.





Because, in general, if we consider that the random variable X is log-normally distributed, then $Y = \ln(X)$ has a normal distribution. Consequently, in our method we did fit the distributions with 2D log-normal distributions as the reviewer was suggesting. We added figures (Fig. S5 in SI) and some more detailed discussion in the Supplementary material to explain our procedure more explicitly.

On the other hand, when we computed the mean and the variance to test the Asymmetric Binomial Partitioning Model we did not compute these on the logarithm of the fluorescence, but on the fluorescence level directly. We can do this because the law of total mean and variance that we used to derive the evolution of both the mean and the variance as a function of the generations is independent from the specific distribution of the data points. Thus it can be used on data from a log normal distribution.

The authors say in the Abstract that the results are “incompatible with an independent division mechanism”, yet their model assumes a binomial partitioning which is based upon independent partitioning. Some clarification on this would be useful.

We agree that the sentence was unclear. The phrase did not refer to the independence between partitioning events as implied by the binomial partitioning model, but referred to the lack of independence on the partitioning of different kinds of organelles in a single partitioning event. Specifically, it referred to the results shown in Figure 4e-f.

In particular, the simultaneous measurement of the partitioning of couples of organelles (i.e MITO-CTV and PKH-CTV) allowed us to compute the correlation of the fluorescence associated to each 2D MITO-CTV or PKH-CTV peak, and to evaluate how this correlation changes from one generation to the next as a result of the partitioning process. The division of mitochondria and cytoplasm exhibits a correlation coefficient of ~ 0.4 , that is preserved during the proliferation, while membrane and cytoplasm have a lower correlation of ~ 0.3 , which is retrieved even if we start with an uncorrelated system (EXP 3).

To further route out the influence of the initial correlation, we compared the experimental correlations with the prediction of the binomial model where the two kinds of dyes segregate independently one from another, and share an arbitrary initial correlation at the population level. As one can see, in all cases the model predicts a progressive decrease of the correlation that is not observed in the data.

As we realized that the phrase in the abstract could be misleading, we have now modified it as “Moreover, we observed that, at each generation, mitochondria and membrane lipids present a stable positive correlation with cytoplasm, which is incompatible with a binomial partition mechanism produced by two independent partitioning processes.” and thank the Reviewer for pinpointing this for us.

The authors do not seem to take into account the fact that (at least) the mitochondria are individual objects and the binomial partitioning should be applied to the organelle, not its fluorescence intensity. A similar point may be made for the cytosol and lipids, though I think it is less important for them.

The Reviewer is basically right. Unfortunately, we are unable to directly count the number of sub-components for each cell (m_g), we are only able to measure the fluorescent intensity (f_g) that is detected by the flow cytometer. Thus we measure the integral of the intensity over time. We assume that the mean and variance are respectively proportional to:

$$E[f_g] \sim aE[m_g] + b \quad \text{and} \quad \text{Var}[f_g] \sim a^2 \text{Var}[m_g]$$

where a is the amount of intensity per unit sub-component, and b is the background fluorescence intensity. In a pure binomial distribution the number of events can be estimated from the *lambda* parameter, but in the partitioning model this is not possible because the contribution to the variance given by the number of particles is small compared to the initial variance of the previous generation distribution and the contribution of p to the distribution. To observe the change of variance due to the number of particles on the measured data we

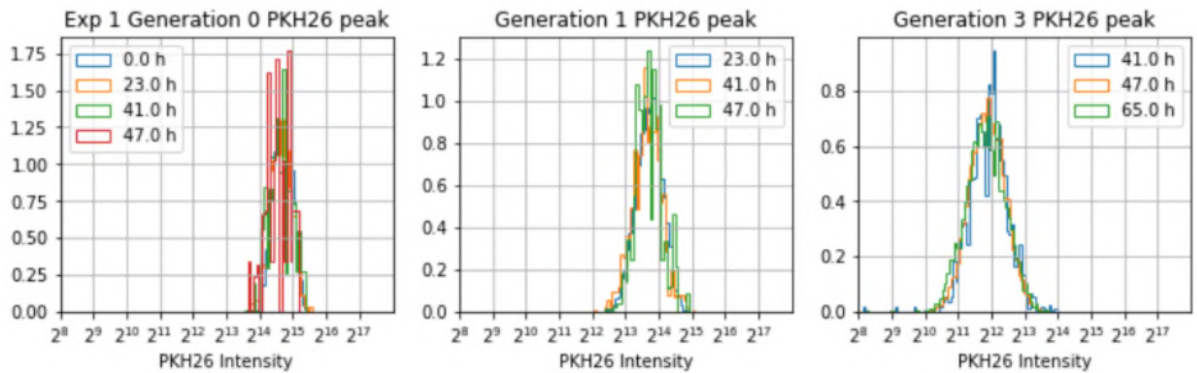
would have to produce a very narrow initial distribution that must be sufficiently small to allow us to observe the subgeneration peaks formed by the asymmetric segregation. But we were not able to get such a narrow peak due to the fact that its width results both from intrinsic and extrinsic factors [2], respectively the instrumental limit for gate width set by the flow cytometer and biological/chemical aspects of the given fluorescence dye. **We added a short discussion of this aspect at the end of Sec. 2.**

[2] Begum, J., Day, W., Henderson, C., Purewal, S., Cerveira, J., Summers, H., Rees, P., Davies, D., & Filby, A. (2013). A method for evaluating the use of fluorescent dyes to track proliferation in cell lines by dye dilution. *Cytometry Part A*, 83(12), 1085–1095. <https://doi.org/10.1002/cyto.a.22403>

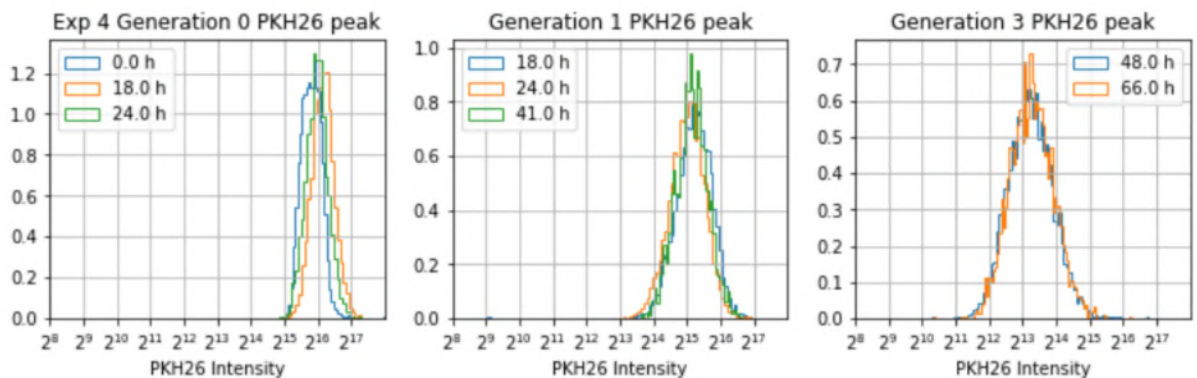
p. 10 penultimate paragraph: I understand the utility of having several markers, where one (the symmetrically partitioning one) helps identifying the generations. However, I do not understand how the authors take into account (or why they do not need to take into account) the loss of fluorescence of the other dye(s) in their model.

It is important to point out that proliferation dyes are optimized to have very little fluorescence loss. Given this, nonetheless, to be sure that the loss of fluorescence of the dyes does not bias our result we have to be sure that the fraction of fluorescence loss is not too large. From our experiments, we observed that the CTV, the PKH26 and the Mito Green had very little fluorescence loss. In general, we observed four different situations.

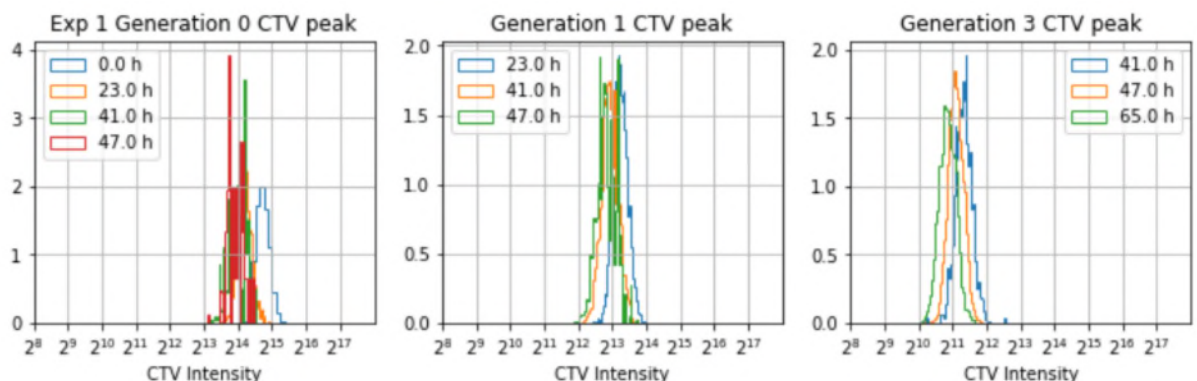
First, especially in Exp 1, the PKH26 dye labeled the cell lipids very effectively and was very stable. We see this in the figure below, which shows the detected peaks from different samples collected at different time points 0, 23, 41, 47 hours. Respectively, each plot shows the detected peak corresponding to a different generation 0, 1, and 3. From these plots we see that the peaks corresponding at a given generation taken from samples collected at different time points perfectly overlap. Thus we can argue that there was a negligible loss-of-fluorescence.



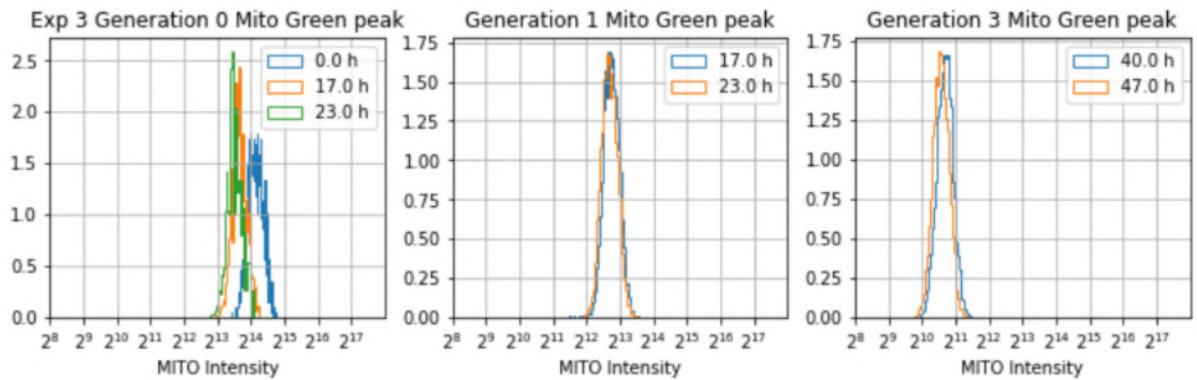
Second, in other experiments the PKH dye showed a very small instability at the beginning of the experiment, in the first hours after the staining procedure. This is shown in the figure below, in which the generation 0 peak average increases from the sample taken at the time point 0 hour to the sample taken at the time point 18 hours. After that it is very stable.



Third, the CTV dye allowed us to make very narrow peaks but the generation peaks tended to slowly decrease in time, with a notably stronger decrease after the time 0. This indicated that a very small part of the CTV dye is degraded in time, and that, similarly to PKH, the CTV presented some kind of instability in the first hours after staining (See figure below).



Fourth, the MitoTracker Green dye showed an initial instability right after the first hours from the staining similarly to CTV, and a much slower loss of fluorescence compared to CTV.



Finally, it is important to notice that from our observation the loss of fluorescence only affects the averages of the peak and that the variances of each peak corresponding to a specific distribution are not affected, or at least they are affected only in a negligible part. Thus since our estimate of the asymmetric partitioning is only dependent on the variance we believe that a loss of fluorescence of this order does not affect our calculation. Clearly a larger loss of fluorescence would have affected our estimate of p , but in that case we would have observed at the very least a major change on the observed peaks variances.

Additionally, changing the fluorochrome did not affect the results indicating that it is not due to the specific characteristic and photostability of each dye used.

Looking at the previous version of the manuscript, we realised that no discussion on the loss of fluorescence was provided. Thus, we have now amended this adding a section in the SI (Sec 3, and Fig. S3 in SI) and a small discussion in the main text.

It would be useful if the authors discuss the influence of different cell cycle lengths among the cells, as well as a potential slowing down of the cycling that may very well be present in their experiments (since they consider up to 5 generations). From my understanding of the work, it should not pose too much of an issue (as long as the generations do not start overlapping) but it would be worthwhile to discuss it.

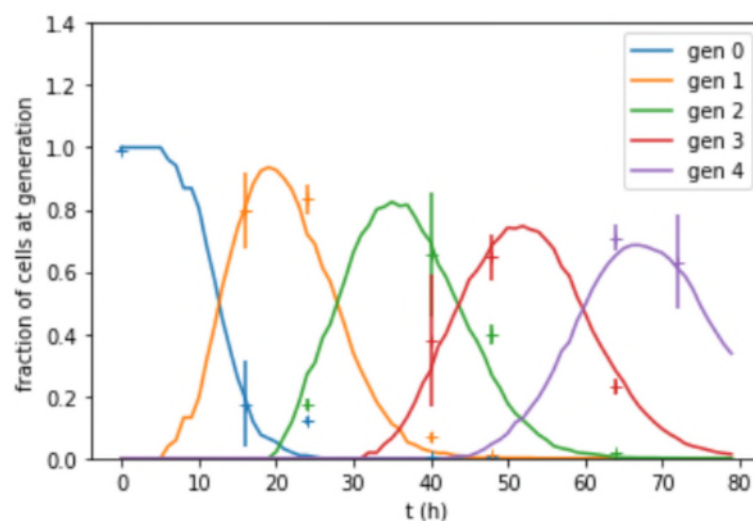
Our model does not assume that cells divide all together and have the same cycle length, but instead it takes into account the possibility of cells with different cycle lengths. Nevertheless, it requires that the fluorescence peak formed by each generation must be detected by the Gaussian Mixture algorithm, and that each peak associated to a certain generation has a measurable variance that is sufficiently constant in time.

As pointed out in [3] if we consider the inheritance of fluorescent particles, and we study how the variance of the total fluorescence distribution changes in time, we see that this is highly irregular because the total fluorescence distribution is composed by mixtures of each generation peak. Consequently,

as time goes by new generations of cells emerge that inherit a fraction of variance and add new peaks to the distribution. In the meanwhile the old generation of cells became neglectable. But since the variance of the inherited distribution of fluorescence for a given generation of cell is fixed, if we can detect the different generation peaks, we are able to account for how this variance changes in time even if cells have different cell cycle lengths.

For this reason we identify the peaks to estimate the generation at which each measured cell belongs. To show that different interdivision times are taken into account by the model, and to show that the proliferation slowing down is negligible, we developed a simulation in which we assumed that the interdivision time for each cell was a random variable drawn according to an Erlang distribution. We used an Erlang distribution because this distribution was found in earlier studies to be a good fit for intergeneration division time (see reference [4]). Furthermore, in the simulation, we imposed from the beginning that cells were synchronized at a certain time as a consequence of the gate strategy. The other assumption of this simulation is that once the interdivision time for a certain cell is passed, the cell produces two new cells, and respectively for each new cell, two new random intergeneration times are extracted to establish when these two cells will divide. Thus, with this simulation we are able to follow in time how the relative frequencies of different cell generations in the population changed. We are also able to plot them in correspondence to the relative generation frequencies obtained from the data with the GMM model at the different time points taken in the experiment dynamics samples. The plot shows a very good correspondence between the measured data and the simulation data obtained with this model. It is important to point out that if we assumed constant interdivision times the fit to the datapoint would not be as good.

Given the match of this simulation with the actual data we obtain, we are confident that the average interdivision time is kept constant as the proliferation progresses and that the population level cell cycle slowing down is negligible.



[3] Lijster, T., & Åberg, C. (2020). Asymmetry of nanoparticle inheritance upon cell division: Effect on the coefficient of variation. *PLOS ONE*, 15(11), e0242547. <https://doi.org/10.1371/journal.pone.0242547>

[4] Yates, C. A., Ford, M. J., & Mort, R. L. (2017). A Multi-stage Representation of Cell Proliferation as a Markov Process. *Bulletin of Mathematical Biology*, 79(12), 2905–2928. <https://doi.org/10.1007/s11538-017-0356-4>

Minor comments:

The title does not reflect the work in my opinion. I think the implication that asymmetric partitioning is responsible for phenotypic variability in cancer cell lines should be removed. As far as I can tell, this is only discussed in the Abstract so obviously no real discussion is provided. If the authors do want to make this case, then I think it needs to be discussed at much more length, because it is not an immediate outcome of the work.

We see the Reviewer's point. Indeed, that phrase referred to an early interpretation of the results which is now surpassed. In fact, although previous research evinced that the partitioning noise impacts on phenotypic heterogeneity and this aspect is worth investigation; our present results can be more directly interpreted in the light of the inheritance of cellular sub-components that were part of the starting population stained at time zero, and how the asymmetric inheritance of this sub-components has consequences with regard to the cancer cell population aging. Indeed, the inheritance of marked organelle, indicates that these organelle were already present in the initial population and are for this reason older organelle. Asymmetric division allows same cells to inherit a larger portion of older organelle, while other cells inherit fewer organelle and are for this reason forced to produce more new organelle before they divide. [5] showed how in Yeast the asymmetric partitioning of mitochondria works as an antiaging mechanism. Thus, we have now removed the reference to phenotypic heterogeneity in the abstract and consolidated the discussion on the aforementioned aspect.

[5] Zhou, C., Slaughter, B. D., Unruh, J. R., Guo, F., Yu, Z., Mickey, K., Narkar, A., Ross, R. T., McClain, M., & Li, R. (2014). Organelle-based aggregation and retention of damaged proteins in asymmetrically dividing cells. *Cell*, 159(3), 530–542. <https://doi.org/10.1016/j.cell.2014.09.026>

Fig. 4e-f. This appears to be, at best, preliminary, with a lack of reproducibility between experiments.

As we already discussed in a previous point, the simultaneous measurement of the partitioning of couples of organelles (i.e MITO-CTV and PKH-CTV) allowed us to compute the correlation of inherited organelles and cytoplasm at each generation.

In particular, the division of mitochondria and cytoplasm exhibits a correlation coefficient of ~ 0.4 , that is preserved during the proliferation, while membrane and cytoplasm have a lower correlation of ~ 0.3 , which is retrieved even if we start with an uncorrelated system (EXP 3).

To further route out the influence of the initial correlation, we compared the experimental correlations with the prediction of the binomial model where the two kinds of dyes segregate independently from one another starting with a correlated initial population. As one can see the model predicts a progressive decrease of the correlation, that is not observed in the data. **We have improved Figure 4 and the relative discussion in Sec. 4.**

Part of the Discussion seems misplaced and is indistinguishable from information covered in the Introduction. This could be consolidated.

We have now streamlined the Discussion as suggested.

The language is in need of a bit of a spell-check before final publication, but it is nothing that prevents understanding of the work.

We have now thoroughly checked the text according to the Reviewer's suggestions. We hope readability has now improved.

Reviewer #2

In their manuscript, Peruzzi et al. develop a FACS-based method to analyze the asymmetry in the distribution of fluorescently-labelled organelles in a proliferating population of human Jurkat T cells. The authors further demonstrate that experimental results obtained on differential organelle distribution can be explained by assuming a minimal model of asymmetric partitioning where the components are divided according to biased binomial statistics. For the most part, the experiments described in this manuscript are well designed and properly executed, and the figures are clearly presented. However, and although interesting, the study falls short in terms of the biological relevance of its conclusions and in representing a significant advance in our present knowledge in the field.

We thank the Reviewer for the appraisal of our work. Regarding the biological relevance, our main objective was to present a new protocol/procedure based on a stochastic dynamical model that can be used in future work to estimate partitioning bias (asymmetric binomial partitioning) in a broad spectrum of biological models. For instance, this method could be used to study asymmetric partitioning of organelle in cancer stem cells or in differentiating cell systems, avoiding costly microscopy time-lapse approaches that are time-consuming and not applicable in some systems.

Nevertheless, reevaluating the experimental results and considering the Reviewers observations, we understood that there is actually a point to be made on cell aging that was not clearly discussed in the former version of the manuscript. Specifically, the asymmetric partitioning of mitochondria and lipidic membrane indicates that there is an uneven division of the mother cell compounds. Hence, for example, if we consider the mito tracker fluorescence distribution for a given generation after some proliferation has occurred, we can deduce that the cells in the low fluorescence portion of the distribution share less mitochondria from the predecessor cells compared to the cells in the high fluorescence portion of the distribution. We thereby conclude that even if Jurkat cells are not cells that tend to differentiate morphologically, they can still use asymmetric division of subcomponents to obtain partial rejuvenation as it has been observed in yeast [5]. We have now expanded the discussion in these terms in the novel version of the manuscript.

Furthermore, as pointed out by Reviewer 2, asymmetric division does not require differentiation but just that the mother cells divide into two cells with different sizes, cellular components and/or fates. Nevertheless, in [6], and also in the papers suggested by the Reviewer, (Izumi and Kaneko, 2012) and (Hwang and Yang, 2016), symmetric division is associated with stemness, and asymmetric division with differentiation. Indeed (Izumi and Kaneko, 2012) and (Hwang and Yang, 2016) are more pertinent because they consider cancer cell lines. In all three of these studies, they show significant asymmetric division in

cells that differentiate, and non-significant asymmetric division (*i.e.* symmetric division) in non-differentiating cells. These works analyze cell populations that present cancer stem cells together with differentiated cells, and show that these cells divide sometimes asymmetrically and sometimes symmetrically. In certain cases (Izumi and Kaneko, 2012), homogeneous cell lines such as Hela cells are shown as examples of symmetrically dividing cells. Here, we consider Jurkat cells which are similar to Hela cells, because these cells are homogeneous cancer cell lines. Furthermore, especially in our standard protocol condition, Jurkats are known to not differentiate. Consequently, given the general perspective of this previous research, we expect these cells to divide symmetrically or present non-significant asymmetric divisions. Nevertheless, our experiments show a residual small degree of asymmetric division, this asymmetric partitioning is clearly much smaller than the asymmetric partitioning discussed in differentiation cells. **Being able to show this may have fundamental consequences on the way we conceptualize symmetric and asymmetric division because it implies that in non-differentiating cell lines asymmetric division persists even if with a small value p . Consequently the finding that we do not get full symmetric $p = 0.5$ divisions raises a biological question.**

To better discuss these points we substantially changed the Abstract, Introduction, and Discussions. For instance, we added to the Introduction the sentence: “Despite its biological importance, the quantification of asymmetry in cellular components at division is often based on microscopy time-lapse approaches that are time-consuming and in some systems not applicable. Furthermore, the division can be tracked only for a few generations and often for only one compound at a time.”

Moreover, we added in the Discussion section: “From a biological point of view, the high resolution of our method allowed us to observe asymmetric partitions in Jurkat T-cell proliferation.

It is worth noticing that even if the asymmetric division was observed in differentiating cells from cancer cell lines and in non-differentiation yeast cells, homogeneous cancer cell lines such as Hela (similar to Jurkat) cells were used as an example of symmetrically dividing cells [Izumi2012].

Being able to show that Jurkat cells preserve a small degree of asymmetric partitioning may have fundamental consequences on the way we conceptualize symmetric and asymmetric division because it implies that in non-differentiating cell lines asymmetric division persists (even if with a small value p).”

- [5] Zhou, C., Slaughter, B. D., Unruh, J. R., Guo, F., Yu, Z., Mickey, K., Narkar, A., Ross, R. T., McClain, M., & Li, R. (2014). Organelle-based aggregation and retention of damaged proteins in asymmetrically dividing cells. *Cell*, 159(3), 530–542. <https://doi.org/10.1016/j.cell.2014.09.026>

- [6] Katajisto, P., Dohla, J., Chaffer, C. L., Pentinmikko, N., Marjanovic, N., Iqbal, S., Zoncu, R., Chen, W., Weinberg, R. A., & Sabatini, D. M. (2015). Asymmetric apportioning of aged mitochondria between daughter cells is required for stemness. *Science*, 348(6232), 340–343. <https://doi.org/10.1126/science.1260384>

Prior analyses already showed that several molecules and organelles are differentially distributed during asymmetric divisions in cancer cells. As such, asymmetric cell division and differential centrosome inheritance have been demonstrated to occur in human neuroblastoma cells (Izumi and Kaneko, 2012). Similarly, the Numb polarity factor seems to control the segregation of subcellular vesicles during the division of colorectal cancer stem cells (Hwang and Yang, 2016). These and other references, in fact, should have been included in the introduction, which somewhat excessively relies on general reviews. Finally, and despite the authors incorporate an asymmetric binomial partitioning model to adjust the experimental data that provides an interesting novel methodological contribution to the analysis of differential organelle distribution, the use of a FACS-based approach to sort out different populations of cells and evaluate organelle partitioning has also been widely used before.

We agree that FACS has already been used before, in fact some of the papers we cited used this technique; our symmetry measurement approach has the advantage of using dyes for live-cell staining that are relatively easy to use as opposite to other methods, such as genetic reprogramming or a transfection system as it is used in [6] and in (Izumi and Kaneko, 2012) and (Hwang and Yang, 2016). These dyes can be used to trace multiple generations of living cells following dye dilution. Thus our method could be used on primary culture and *in-vivo* studies which are considered to be promising improvements on the traditional *in-vitro* studies [7]. We have now both made this point clearer in the introduction and improved the bibliography according to the Reviewer suggestions.

We have now included in our manuscript the research proposed by the Reviewer and also modified our discussion, to better represent the context that allows us to interpret our result, as already discussed in the response above.

In fact, we would like to stress that although asymmetric partition was observed in differentiating cells from cancer cell lines, and asymmetric partition was observed in non-differentiation yeast cells, asymmetric partitioning was not reported for non-differentiation homogeneous cancer cell lines. On the contrary, homogeneous cancer cell lines such as Hela cells were used as an example of symmetrically dividing cells.

- [7] Wilding, J. L., & Bodmer, W. F. (2014). Cancer cell lines for drug discovery and development. In *Cancer Research* (Vol. 74, Issue 9, pp. 2377–2384). American Association for Cancer Research Inc. <https://doi.org/10.1158/0008-5472.CAN-13-2971>

Besides these general considerations, other main issues regarding the manuscript are:

1.- The authors analyze the partitioning of cytoplasm, cell membrane and mitochondria during the proliferation of Jurkat lymphocytes derived from human T cell leukemia. Their results suggest that while in Jurkat cells the cytoplasm is evenly partitioned between the daughter cells, asymmetric distribution of membranes and mitochondria can be observed. However, a main objective of the manuscript was to help elucidating whether variability associated to the asymmetric partitioning of cellular components could play an important role in tumorigenesis. The authors could have thus exploited the designed experimental and computational protocol to dig deeper into this matter. In this way, by using their methodology to follow the distribution of organelles in populations of cells during mitosis in a relatively fast and easy manner, the authors could have evaluated whether there exist differences in the partitioning of cytoplasm, cell membrane and mitochondria during the proliferation of Jurkat T cells and in populations of immortalized non-transformed T cells, such as TERT-expressing T cells. Since TERT-expressing T cells do not develop genetic changes characteristic of malignant transformation and maintain intact the mechanisms that regulate growth (Barsov, 2011), this comparison between transformed and non-transformed cells could thus reveal differences in asymmetric partitioning of organelles that might shed light on whether their differential distribution had a possible role of in tumor evolution.

The comparison, the Reviewer is suggesting, seems a very promising piece of work. Indeed we are working on the application of our novel strategy to other cell systems that may be of more direct interest from a biological/medical point of view. In fact, we agree that Jurkat cells are a cell line used mostly as a model system for cancer investigation, thus our aim is to apply our apparatus to the study of asymmetric organelles partitioning to more physiological systems and so better understanding the division process that takes place in a transformed cell compared to a normal one.

However, understanding if the variability associated to asymmetric partitioning is associated with tumorigenesis is not a main objective of the present work, - in which we developed and tested our technical and theoretical apparatus on a biological model system - but a downstream application that we want to pursue in time. The point that we want to make is that with our method we can measure even very small asymmetry parameters p because using flow cytometry we can measure a large number of cells. Furthermore, given that in our experimental conditions Jurkat cells are not changing their morphology and are not differentiating, we wanted to show that even in this condition a small asymmetric partitioning parameter p is preserved. This observation is in contrast to previous research (Katajisto *et al.*, 2015), (Izumi and Kaneko, 2012), and (Hwang and Yang, 2016) which associated asymmetric division only with differentiation cells and showed that cells that are not changing their morphology or differentiating have negligible asymmetric partitioning.

We have now added a comment on the future objectives of our method and thank the Reviewer for the insightful comment.

“Finally, we want to stress that our apparatus can be easily applied to different cell lines and different kinds of compounds providing a powerful tool for understanding partitioning-driven heterogeneity. Moreover, our method is not based on transfection, and therefore in the future, it may be used in primary cell cultures.

Hence, our method could be used on primary culture and *in-vivo* studies which are considered to be promising improvements on the traditional *in-vitro* studies (Wilding, 2014). For instance, in future work, we could evaluate whether there exist differences between the partitioning of Jurkat T cells and of non-transformed T cells, for which the mechanisms that regulate growth are unaltered [Barsov2011]. These kinds of comparisons could help us understand whether differences in partitioning play a role in tumor evolution.”

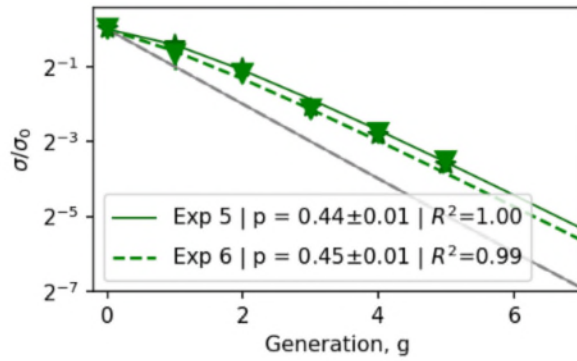
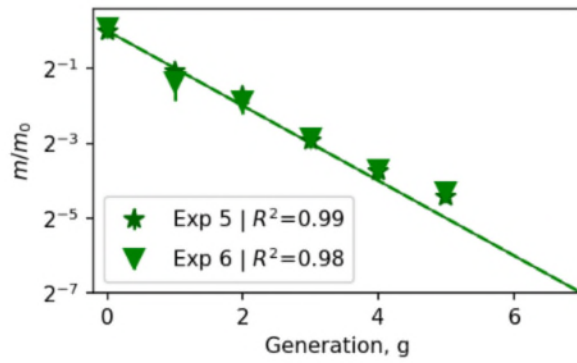
2.- The dyes used to mark the cell membrane, cytoplasm and mitochondria have different fluorescent properties and photostabilities. This is a factor that could definitely have an impact on the outcome of the FACS analyses. Since cell markers coupled to different fluorophores are commercially available, the authors could have easily performed the same experiment with a different combination of dyes (e.g., CellTrace™ Yellow, MitoTracker™ Red FM and PKH67 green fluorescent cell linker kit for general cell membrane labeling) as a control for the methodology.

First of all, we thank the Reviewer for suggesting this important check of the methodology.

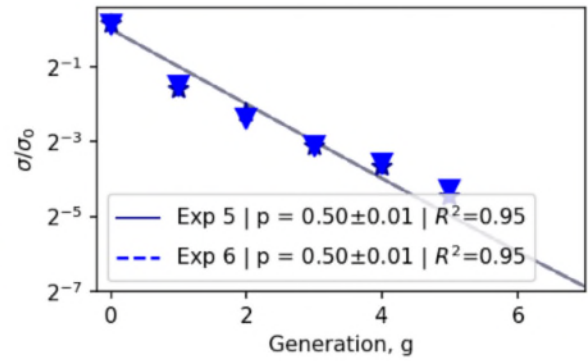
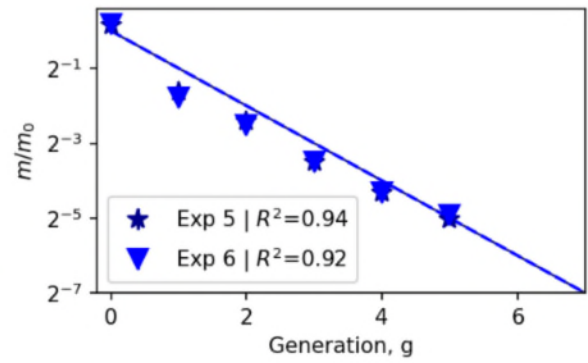
To confirm the data and to avoid artefacts due to the different fluorescent photo-stabilities and properties, we performed additional experiments using different dye combinations to mark the cell membrane, cytoplasm and mitochondria. In particular, we used CellTrace™ Yellow (C34573 A, Life Technologies, Paisley, UK) and CellTrace™ Far Red (C34572, Life Technologies, Paisley, UK) to mark the cytoplasm; MitoTracker® Deep Red 633 FM (M22426, Molecular Probes, Eugene, USA) and PKH67 green fluorescent cell linker (MINI67-1KT, Merck Life Science, Milano, Italy) to mark mitochondria and membrane proteins, respectively.

As one can see from the plot below, changing the dyes does not alter the partitioning process. In fact, both the mean and variance trends are still compatible with a binomial model with mitochondria and membrane proteins dividing asymmetrically, while the cytoplasm shows an even division.

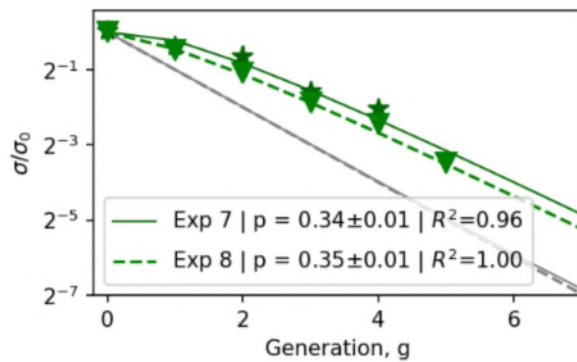
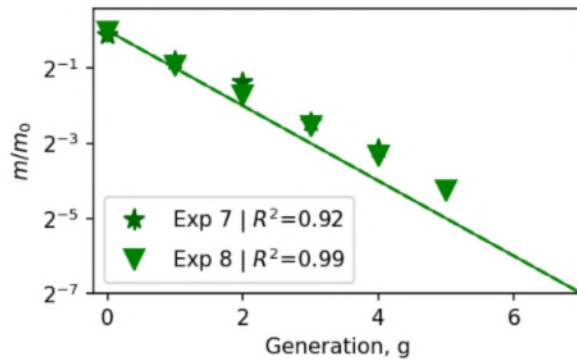
MITOCHONDRIA [Deep Red 633 FM]



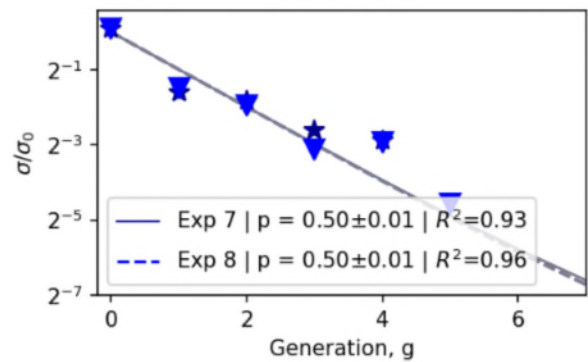
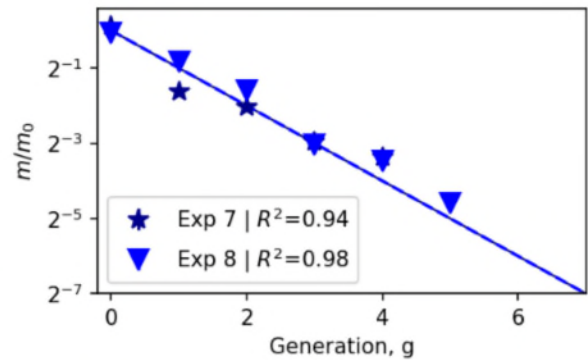
CYTOPLASM [CTY]



MEMBRANE [PKH67-green]



CYTOPLASM [exp 7: CTY, exp 8: CTFR]

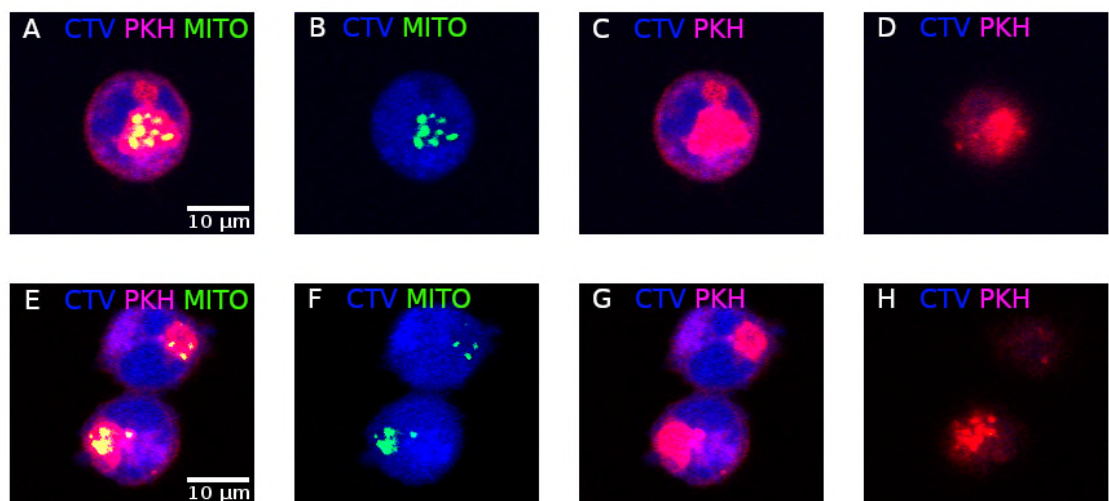


We have now added the plots of the new experiments in the SI and a short discussion in the main text (Fig. S7 in SI). Nevertheless, as discussed in a

previous response to Reviewer 1, the major problem of most of these dyes is that they present an instability in their expression of the fluorescence intensity in the first hours of the experiments that makes the measurement of the peak associated with generation 0 in certain cases more problematic. The initial selection of dyes is the one that has presented smaller initial instability.

3.- Peruzzi et al. speculate that asymmetric cell membrane partitioning may be linked to aggregated of membrane proteins formed after T cell activation. In order to verify this hypothesis, the authors acquired confocal microscopy images that they indicate that showed aggregation clusters at the membrane level for the PKH fluorescence in many cells (page 10, line 217). However, these images are not shown. The authors should either include the confocal images or remove this statement. Likewise, when discussing the results from Figures 2c-d, it is indicated that the same behavior is observed when the mitochondrial marker is used instead of the PKH maker, but the data is not shown (page 6, line 126). These results should be included as supplementary data.

We see the Reviewer point. We have added both the confocal images (below) and the analog of Figure 2 for MITO-CTV data in the novel version of the SI.



These confocal microscopy Images show representative data for the CTV (blue), Mito (green) and PKH (red) multiparametric staining(A and E). To better visualize each compartment, coupled stainings are imaged for cytoplasm and mitochondria (B-F) or cytoplasm and membrane (C-G). Representative confocal images taken at the top of the cell to show membrane protein aggregates for PKH (in red) are displayed in panel D and H. This figure was added to SI, see Fig. S9.

Finally, some minor concerns are:

1.- The text should be reviewed for proper syntax and word choice. Results should be written in past tense. Finally, there are also several typos (e.g., “cellular component” instead of “cellular components” (page1, line 2), “PHK” instead of “PKH” (page 3, line 36), “fitt” instead of “fit” (page 4, Figure 2 legend), etc.).

We have now thoroughly revised the text according to the Reviewer's suggestions. We hope that readability has now improved.

2.- The definition of asymmetric cell division in the introduction is not accurate. In general, an asymmetric cell division results in two cells that differ in size, cellular components and/or fate, but does not necessarily ends up with one of the cells differentiating into a specific cellular type.

The Reviewer is completely right. We have now fixed the definition. This definition is actually much more helpful and it allows us to explain our results more clearly. Indeed, the asymmetric partition of mitochondria results in asymmetric division of cells with different shares of progenitor mitochondria.

We have added a new paragraph to the introduction explicitly stating this definition: “At each division, an initial cell often referred to as the mother, first duplicates its genetic material and cellular components, then it halves into two daughter cells. Depending on how the internal components are partitioned, we may have a symmetric division if the daughters are identical to the mother, while we speak of asymmetric cell division when the two daughter cells differ in size, cellular components and/or fate.”

3.- The figure legends are, in many cases, missing important information. In this way, while in Figure 2 it is indicated what do the dark blue, red and green dots represent, no information is provided about the light blue and yellow dots. Likewise, it should be clearly stated what do the star, triangles and circle represent in Figure 4. These symbols are referred to as “dots”, which leads the reader to confusion. Finally, in the legend for Figure 4, the statement “the results are very consistent” could be removed. This is a consideration that, if necessary, could be introduced in the text.

We have now fixed the legends and thank the Reviewer for the suggestions.

4.- In Figure 2b, percentages of cells in each generation are reported in an insert as bar plots. However, no scale for the plots is provided.

We have now modified the figure adding the scale as suggested.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

In line with my previous review, I feel the method presented in the manuscript is of interest for future work in the field. The revised version is also more convincing in the instances where I felt more evidence or argument was warranted. In some cases, I give some further suggestions below, but also when not explicitly mentioned, I consider that the authors have addressed my concerns. Overall, I do not see any reason this should not be accepted. In addition, I offer some further suggestions below, but they are more issues of clarity and phrasing, rather than scientific concerns.

The authors have, largely speaking, addressed my comment about the selection of a narrow initial population potentially biasing the results. I wish they would have discussed it more clearly, separating general (potential) issues from the effect on their actual experiments. I also wish they had *not* discussed the issue under the assumption that one either has to synchronise the cells or select a narrow initial population, because I think that is an unnecessary restriction (other options exist, though admittedly with their own disadvantages, and they are even referenced in the paper). Finally, I wish the main outcomes of this discussion would have made it to the main text, rather than being relegated to the supplement. Nevertheless, there is certainly enough mentioned to highlight this issue for readers of the paper and there does not appear to be any effect on the results the authors present, so I consider this addressed.

I still think it could be clearer in the manuscript that the Gaussians are applied to the log of the data (and hence that my suggestion that the distribution looks more log-normal than normal is entirely consistent with what the authors have assumed). However, this is a phrasing and clarity issue, rather than a scientific one. Furthermore, the authors have clarified the extraction of the parameters, which, indeed, I seemed to have misunderstood in the previous version.

With regards to my point about different cell cycle lengths, the authors appear to have misunderstood this point in their rebuttal. I do not think there is an issue (which I feel was clearly stated), but I do think it is a point worthwhile to discuss in the manuscript. Indeed, I would even consider the fact that the authors' method does not depend (heavily) upon a constant cell cycle length an advantage. I thus still hold the opinion that this is something worthwhile discussing (briefly) in the manuscript, and not just the rebuttal.

Minor comments:

p. 4 "showing low toxicity": This makes it sound as if the cells are not toxic. I assume what the authors are trying to say is that the cells show a low toxic *response* to dyes.

p. 4 "cells stained for live-dead compounds were highly viable (see Figure S4...)": This makes it sound as if the authors have checked viability using a live/dead assay and that it will be presented in Fig. S4. In reality, Fig. S4 presents a gating strategy that to me has very little to do with this.

p. 4 "Unexpectedly, narrow sorting of stained cells": It is not clear to me in which sense this is unexpected. Indeed, to me it is fully expected and, I suspect, it is also for the authors, because it is the whole basis of the work.

p. 4 “t is important to point out that the synchronization is a result of the sorting step, but in general, our method does not require synchronization”: It is unclear to me what the authors are trying to say here. Read in context, it seems to say that their method could be performed without the initial sorting step, which I believe is false (if the authors disagree, then they need to show evidence that it works also without this step). However, what I think the authors are trying to say is that their method does not require synchronisation in a real sense, only synchronisation in the sense of a narrow sorting. In essence, the confusion arises because the authors use the word synchronisation to refer also to the act of sorting to achieve a narrow initial population. I would urge the authors to rephrase for clarity.

p. 4 paragraph starting on line 104: The whole discussion is made in terms of base-2 logarithms and then ends with a reference to a figure in base-10 (Fig. 1d).

p. 5 “we measure the integral of the intensity over time”: It is not clear to me what the authors mean by this (the same goes for their rebuttal). A generous interpretation could be that they measure the convolution of the number of units per cell over the fluorescence expected for a given number of units, but that is most definitely not what is written. Having said that, I do agree with the authors’ point in the rebuttal that there is no issue.

p. 5 line 119: $E[fg]$ should be $E[f_g]$, I believe.

p. 5 line 133 “cell tuples and fluorescence debris”: “cell doublets and debris”

p. 5 “the Pearson correlation between the two intensities appears to remain constant [...] as shown in Figure 2e”: I am unsure of how the authors consider the results in Fig. 2e to be constant, as they show significant variation. If they mean to imply that the correlation remains low throughout, then I think there are better ways of phrasing that and I would also adjust the graph (i.e., change the y axis to range between 0 and 1) to better show this.

p. 9 “lines show the predictions of the asymmetrical binomial model”: This is true, but a bit misleading. A simple exponential decay gives the same prediction. In other words, it does not really show the suitability of the model per se (later results do, however) which is how it comes across.

p. 11, line 330: “Hela” should read “HeLa”

Some references are missing article numbers or page numbers. For instances: ref. 8, 16-17, 58 and 62 (I didn’t check all, but happened to notice these).

Fig. S4. Please write out what FV780 is.

The language is still in need of a bit of editing before final publications, but as before, it is nothing that prevents understanding of the work.

Reviewer #2 (Remarks to the Author):

In this revised version of their manuscript, Peruzzi et al. provide a series of arguments and new

evidence to reinforce the conclusions drawn in the original version of their work and to clarify the main issues raised during the review. I would like to acknowledge the notable effort that the authors have done, overall, in order to answer to most of my concerns. In this sense, Peruzzi et al. have successfully replied to most issues regarding the methodology, including new additional data that more strongly support their FACS-based experimental and computational approach to evaluate the asymmetric partitioning of cellular components during the division of Jurkat cancer T cells. The authors have nonetheless not tackled the other main issue raised during the revision, which had to do with the biological implications of their results. Without further exploiting their methodology to dig deeper into whether the differential distribution of cellular organelles could have a possible role in tumor evolution, the data presented does not provide enough strong evidence supporting such a possibility. Despite the main objective of the manuscript was to develop a new protocol to estimate partitioning bias, having used it to get new insights on such an exciting biological question would have definitely increased its interest and potential impact. On a final note, I also consider that the redaction of the text could be still significantly improved.

Reviewer #1

Remarks to the Author:

In line with my previous review, I feel the method presented in the manuscript is of interest for future work in the field. The revised version is also more convincing in the instances where I felt more evidence or argument was warranted. In some cases, I give some further suggestions below, but also when not explicitly mentioned, I consider that the authors have addressed my concerns. Overall, I do not see any reason this should not be accepted. In addition, I offer some further suggestions below, but they are more issues of clarity and phrasing, rather than scientific concerns.

The authors have, largely speaking, addressed my comment about the selection of a narrow initial population potentially biasing the results. I wish they would have discussed it more clearly, separating general (potential) issues from the effect on their actual experiments. I also wish they had **not** discussed the issue under the assumption that one either has to synchronise the cells or select a narrow initial population, because I think that is an unnecessary restriction (other options exist, though admittedly with their own disadvantages, and they are even referenced in the paper). Finally, I wish the main outcomes of this discussion would have made it to the main text, rather than being relegated to the supplement.

Nevertheless, there is certainly enough mentioned to highlight this issue for readers of the paper and there does not appear to be any effect on the results the authors present, so I consider this addressed.

We thank the Reviewer for having appreciated our effort in the replies. We have now added the outcomes of the analysis on the narrow initial population selection in the Main Text.

I still think it could be clearer in the manuscript that the Gaussians are applied to the log of the data (and hence that my suggestion that the distribution looks more log-normal than normal is entirely consistent with what the authors have assumed). However, this is a phrasing and clarity issue, rather than a scientific one. Furthermore, the authors have clarified the extraction of the parameters, which, indeed, I seemed to have misunderstood in the previous version.

We have now rephrased it and explained the point more clearly.

With regards to my point about different cell cycle lengths, the authors appear to have misunderstood this point in their rebuttal. I do not think there is an issue (which I feel was clearly stated), but I do think it is a point worthwhile to discuss in the manuscript. Indeed, I would even consider the fact that the authors' method does not depend (heavily) upon a constant cell cycle length an advantage. I thus still hold the opinion that this is something worthwhile discussing (briefly) in the manuscript, and not just the rebuttal.

We apologize for the misunderstanding. We have now added a short discussion on the cell cycle in the manuscript.

Minor comments:

p. 4 “showing low toxicity”: This makes it sound as if the cells are not toxic. I assume what the authors are trying to say is that the cells show a low toxic *response* to dyes.

The Reviewer guessed right. We have now amended the text.

p. 4 “cells stained for live-dead compounds were highly viable (see Figure S4...”: This makes it sound as if the authors have checked viability using a live/dead assay and that it will be presented in Fig. S4. In reality, Fig. S4 presents a gating strategy that to me has very little to do with this.

We have now explained the content of the plot more clearly in the caption as we realized that it was not described in detail.

The first panel of Figure S4 (top-left) shows a scatter plot of cell size (FSC-A) vs FVS780, that is the Fixable Viability Stain 780 dye used to mark dead cells. As you can see, 96% of the cells are FVS780 negative, which indicates high cell viability. Whereas, the other plots represent the following gating strategy for cell isolation.

p. 4 “Unexpectedly, narrow sorting of stained cells”: It is not clear to me in which sense this is unexpected. Indeed, to me it is fully expected and, I suspect, it is also for the authors, because it is the whole basis of the work.

The Reviewer is right; we have now removed the word ‘Unexpectedly’.

p. 4 “it is important to point out that the synchronization is a result of the sorting step, but in general, our method does not require synchronization”: It is unclear to me what the authors are trying to say here. Read in context, it seems to say that their method could be performed without the initial sorting step, which I believe is false (if the authors disagree, then they need to show evidence that it works also without this step). However, what I think the authors are trying to say is that their method does not require synchronisation in a real sense, only synchronisation in the sense of a narrow sorting. In essence, the confusion arises because the authors use the word synchronisation to refer also to the act of sorting to achieve a narrow initial population. I would urge the authors to rephrase for clarity.

We see the Reviewer point. We have now rephrased the sentence more clearly.

p. 4 paragraph starting on line 104: The whole discussion is made in terms of base-2 logarithms and then ends with a reference to a figure in base-10 (Fig. 1d).

We have now corrected the scale in the plot of Fig. 1d.

p. 5 “we measure the integral of the intensity over time”: It is not clear to me what the authors mean by this (the same goes for their rebuttal). A generous interpretation could be that they measure the convolution of the number of units per cell over the fluorescence expected for a given number of units, but that is most definitely not what is written. Having said that, I do agree with the authors’ point in the rebuttal that there is no issue.

We measure the fluorescence intensity, f_g as the integral of the fluorescence signal collected over the time while the cell is exposed to the laser beam.

p. 5 line 119: $E[fg]$ should be $E[f_g]$, I believe.

Done.

p. 5 line 133 “cell tuples and fluorescence debris”: “cell doublets and debris”

Done.

p. 5 “the Pearson correlation between the two intensities appears to remain constant [...] as shown in Figure 2e”: I am unsure of how the authors consider the results in Fig. 2e to be constant, as they show significant variation. If they mean to imply that the correlation remains low throughout, then I think there are better ways of phrasing that and I would also adjust the graph (i.e., change the y axis to range between 0 and 1) to better show this.

Yes, we meant that the correlation remains at a low non-zero value. We have now changed the graph axis and rephrased the text accordingly.

p. 9 “lines show the predictions of the asymmetrical binomial model”: This is true, but a bit misleading. A simple exponential decay gives the same prediction. In other words, it does not really show the suitability of the model per se (later results do, however) which is how it comes across.

We have now rephrased the sentence more clearly.

p. 11, line 330: “Hela” should read “HeLa”

Done.

Some references are missing article numbers or page numbers. For instances: ref. 8, 16-17, 58 and 62 (I didn’t check all, but happened to notice these).

Checked.

Fig. S4. Please write out what FV780 is.

Done.

The language is still in need of a bit of editing before final publications, but as before, it is nothing that prevents understanding of the work.

We have now further revised the manuscript to improve the English.