Dear Dr. Manoj Kumar Jana,

Thank you very much for your time and editorial feedback on our manuscript which was greatly appreciated during our revisions.

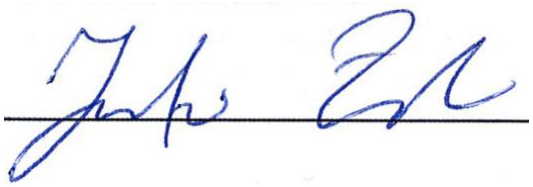
We also sincerely appreciate the time taken by the reviewers to read our manuscript and kindly thank them for their valuable comments and suggestions. In addressing the suggestions and comments closely, we feel that the manuscript is significantly improved. Our detailed responses to each of the reviewers' comments are provided below. For the reviewers' and your convenience our responses are written in blue font colour.

We would like to highlight the new data that we have included in the manuscript to address Reviewer 2's concerns around the effect of inactive PST-1P on microtubule dynamics: 1) a new **Supplementary Figure 1** showing blastocyst formation in embryos treated with inactive PST-1P as compared to control embryos and 2) normally dividing embryos in both conditions (**Supplementary Figure 2**). We agree that this was an important aspect to prove and we belief that our new data convincingly show that inactive PST-1P does not impair embryo quality or microtubule dynamics.

We would also like to clarify that the manuscript text has been amended to consistently use the abbreviated form of units for seconds (s), minutes (min) etc as requested. However, in instances where these terms are not used as a quantified amount, we wish to keep the full form of the word. For example, when referring to "sub-second response times" (line 102).

All other modifications are related to the editorial or reviewers' comments as outlined below.

Best regards,



Dr. Jennifer Zenker

Editorial comments:

Editorial comments to Authors:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We thank you for this recommendation and have taken the opportunity to check for and correct any spelling or grammatical issues.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have revised the manuscript to remove all personal pronouns and have replaced these with alternate wording.

3. Please provide an email address for each author.
We have provided an email address for each author on the first page of the manuscript.

4. Please provide at least 6 keywords or phrases.
The six keywords we wish to provide are included on the first page of the manuscript.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (e.g., NanoDrop, Zeiss LSM780, Zeiss ZEN Black, etc)
We have removed all commercial language and instead have referred to these products with generic terms. We have added details of the commercial suppliers into the Table of Materials.

6. Please ensure that all units are abbreviated throughout the manuscript: L, mL, µL, cm, kg, etc. Use h, min, and s for hour, minute, and second, respectively.
We have checked the manuscript thoroughly to ensure all units are written as prescribed and abbreviations are used consistently throughout the manuscript.

7. Please number the headings within protocol and adjust the numbering of the steps accordingly.
We have replaced the headings within the protocol with numbers as advised.

8. 2.8 and 2.9: Please provide more details of how the embryos were transferred.
We have adjusted the text in 2.8 and 2.9 to clarify that the embryos are transferred by mouth pipetting:

“2.8 Transfer embryos by mouth pipetting into the PST-1P-treated KSOM droplet from Step 2.7.

2.9 Immediately transfer embryos by mouth pipetting into the centre of the PST-1P-treated KSOM droplet in the imaging chamber slide, prepared in Steps 2.4 – 2.6 (Figure 1A).”

9. 2.10: Please provide the conditions (temperature, humidity, atmosphere, etc.) used in the environmental chamber.
We have adjusted the text in 2.10 to the following:

2.10 Allow embryos to incubate at 37 °C, 5 % CO2 in PST-1P-treated KSOM in the imaging chamber slide for at least 1 h prior to imaging. If possible, mount the chamber slide on the microscope inside an environmental chamber at 37 °C, 5 % CO_2 and in complete darkness to ensure all PST-1Ps are in the inactive trans-configuration and that embryos can sink to the bottom of the dish.

10. Figure 3, legend: Please include the full form of DIC.
We have amended the figure legend for Figure 3 to include the full form of DIC (Differential interference contrast).

11. Please ensure that every figure panel showing a microscope image includes a scale bar
We have added scale bars to each panel of figures including a microscope image.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.
We have amended the reference list in line with this advice.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This protocol presents a detailed guide to establishing photocontrol over microtubule dynamics in the preimplantation mouse embryo, a challenging setting due to its 3D nature and the zona pellucida.

Major Concerns:
None.

Minor Concerns:
Text is clear and well-written. Recommend accept with minor text changes as follows:

line 63: "light-activated" should be e.g. "first irreversibly light-activated (photocaged), then more recently, reversibly light-activated (photoswitchable)". and line 65 adapted accordingly (eg reversibly photoswitchable PST). Since light-activated by itself means photouncageable (which has been done since the 1970s), not photoswitchable.

We thank reviewer 1 for this very helpful comment. We have updated lines 84 – 88 according to the suggestion made by the reviewer:

"The development of light-activated compounds began with the creation of photouncaged molecules and has heralded a new era in targeting and monitoring the effects of microtubule growth inhibition in a precise and spatiotemporally-controlled manner. One family of reversibly photoswitchable drugs, the photostatins (PSTs), were developed by replacing the stilbene component of combretastatin A-4 with azobenzene[8]."

Same issue at line 387, line 390: "photoswitchable" not photoactivatable.

We have changed line 387 – 390 (now lines 419 – 421) from "photoactivatable" to "photoswitchable".

line 69: "Cis-PSTs are reversible inhibitors" in what way reversible (important?)? just clarify for the new reader.

line 69 also: important to state here already that "whereby the inactive trans-configuration converts to the active cis-configuration by isomerisation;"

We thank reviewer 1 for this comment and have updated the text now on line 90 to: *"PSTs are inactive until illumination with UV light, whereby the inactive trans-*

configuration converts to the active cis-configuration by reversible isomerisation. Cis-PSTs are inhibitors of microtubule polymerisation…"

line 92: "similarly, other light-reactive" - this example is a photouncaged compound, not sure this is really similar.

We have deleted "similarly" from line 92 (now line 114).

lines 239-240, 245: is this pixel resolution? pixel count, density, etc? Also "120 frames" not time frames.

Yes, it is pixel resolution described now on lines 255 and 265. We wish to keep "time frames" because we find it more precise than "frames" as this could refer to z-frames as well.

line 432: "... is essential, a new class of..." - delete "the development of" (they are published & preprinted) and add a reference to the preprint with the optimised structures (https://dx.doi.org/10.1101/2021.03.26.437160)

We have deleted "the development of" from line 466 (previously line 432) and added reference to the preprint as suggested by reviewer 1 (Reference 25).

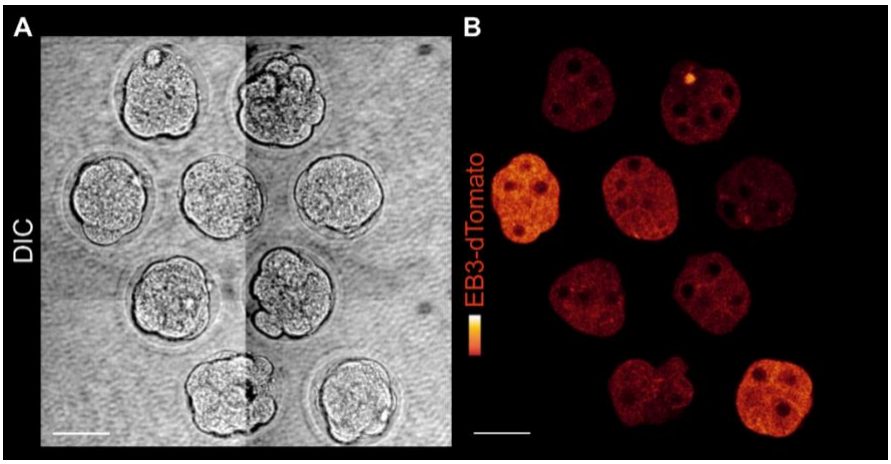
Reviewer #2:

Manuscript summary:

This is a potentially interesting and useful protocol for embryo biologists for photo-controlled MT depolymerisation. Please see my concerns below. Overall i am somewhat concerned based on some of the images shown that the method is not as clean and user friendly as it is made to sound.

In lines 294-295, the authors conclude that '…PST-1P does not elicit inhibition of microtubule polymerisation under dark conditions…'. This statement is not well supported by the data presented. Despite all the precautionary steps taken to avoid inadvertent PST-1P activation, microtubule dynamics appears different between control and inactive PST-1P treated embryos (Fig. 2Ai vs. 2Bi) even before the activation step.

We thank reviewer 2 for this comment. A comparison of the 3D images of control and PST-1P treated embryos in Figure 2Ai and Bi shows a slightly higher overall fluorescence signal in the control embryo. However, this is due to a higher level of background signal in control embryos, which can vary widely between embryos. In support of this, we have provided here an example of untreated embryos collected on the same day and kept under identical culture conditions to demonstrate the variability of EB3 fluorescence intensity between embryos (see image below; not included in manuscript). Variability between embryos is an inevitable caveat when working with physiological live-cell models.



Expression of EB3-dTomato in untreated control embryos. Tile scan of the (A) DIC and (B) EB3-dTomato signal in 16-cell stage untreated preimplantation mouse embryos isolated, microinjected, cultured and imaged pooled together at all times, showing variability in expression of EB3-dTomato. Scale bars are 50 µm.

In this protocol, we describe the use of preimplantation mouse embryos which have been microinjected with cRNA encoding for fluorescently tagged EB3 at the 1-cell stage. This RNA is translated by the embryo and expression levels can vary widely between embryos by the 8-16-cell stage, even in untreated embryos collected from the same mouse and cultured in identical conditions. In addition to variation in

expression of the construct, the signal intensity of fluorescently-tagged proteins becomes diluted and degraded with each subsequent cell division.

As a marker of microtubule polymerisation, some cell cycle-related fluctuation in EB3-dTomato signal can be expected as preimplantation embryos undergo multiple rounds of cell division in fairly rapid succession. This can result in slight variation of cell cycle timing between embryos and even between cells in the same embryo.

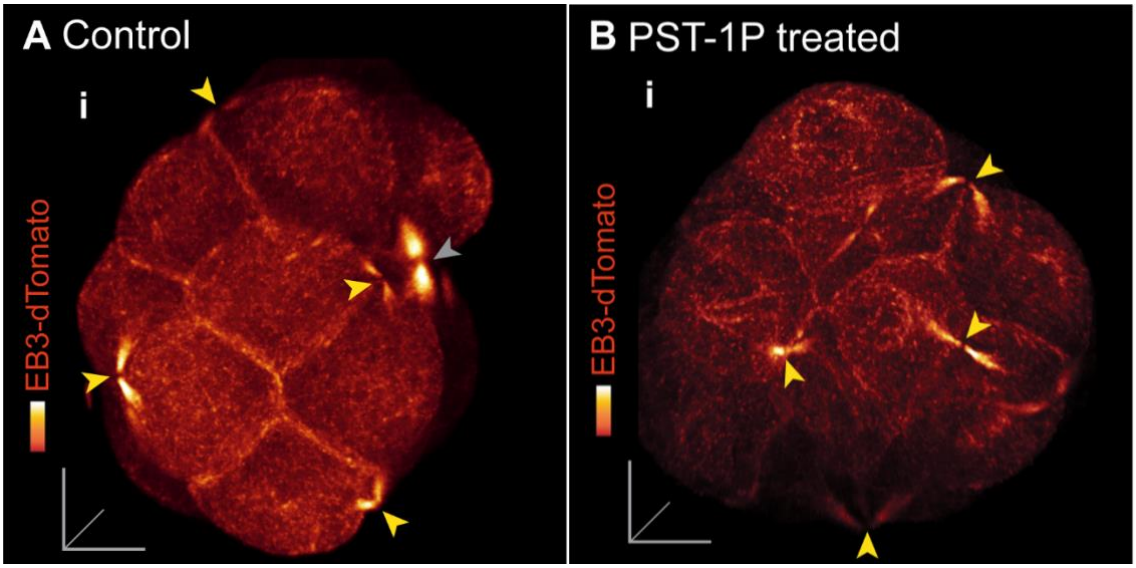
While every measure is taken to ensure comparability of EB3-dTomato expression between control and PST-1P treated embryos, some variation is unavoidable, which is a known limitation of using exogenously expressed constructs in live organisms such as the preimplantation embryo.

We also wish to kindly refer to the insets of the same embryos in Figures 2Aii and 2Bii where there is a similarity in EB3-dTomato signal prior to activation in control and PST-1P treated cells. While lower resolution images shown in Figures 2Ai and 2Bi (one scan = 5 minutes) may appear slightly variable in overall intensity, the specific comet-like signal of EB3-dTomato can only be properly visualised by zooming in and with fast temporal scanning of a ROI. This is best shown in Figures 2Aii and 2Bii (one scan = 0.5 seconds), which demonstrate similar EB3-dTomato signals in control and PST-1P treated embryos prior to activation.

The overall level of EB3-dTomato signal seems lower and the microtubule bridges look thinner in PST-1P treated embryo prior activation. In light of this concern the authors should therefore provide independent evidence that MTs polymerisation is not invertedly affected by PST-1P treatment prior activation. The authors could compare chromosome segregation error rates and show that the spindle dynamic between control and inactive PST-1P-treated embryos remains unchanged.

We thank reviewer 2 for this excellent suggestion. The differences in the thickness of the interphase bridges in control and PST-1P treated embryos in Figure 2Ai and 2Bi can be explained due to the narrowing of the bridge following the cessation of the mitotic cell cycle. A wider microtubule structure can be observed in Figure 2Ai, located at the right-hand side of the embryo (indicated below by a grey arrowhead). This represents a cytokinetic bridge, rather than an interphase bridge and the reviewer is directed to the interphase bridges (indicated by yellow arrowheads), which appear comparable between the control and PST-1P treated embryos. Furthermore, the position, size, and shape of interphase bridges are highly variable, as demonstrated by Zenker et al. (Zenker et al. 2017, *Science*). We have also added these arrowhead

annotations to Figure 2 in the manuscript and updated the figure legend to describe the indicated bridges.



Control and PST-1P treated embryos showing various stages of microtubule bridge maturation. (A) Control and (B) PST-1P treated embryos showing cytokinetic (grey arrowhead) and interphase (yellow arrowhead) microtubule bridges (EB3-dTomato). Adapted from Figure 2 in manuscript

We agree with reviewer 2 that microtubule polymerisation dynamics should not be affected by PST treatment under dark conditions. However, chromosome segregation errors occur frequently under normal conditions during mammalian preimplantation development. These errors do not necessarily affect embryonic development and this is an active field of research to understand this impressive plasticity of the mammalian embryo (Mashiko, Ikeda et al. 2020). Nonetheless, real-time imaging to visualise the spatiotemporal aspects of mitotic spindles in a dividing embryo is a useful approach to show no significant effects of inactive PST-1P on microtubule dynamics. Therefore, we provide a new supplementary figure (Supplementary Figure 2) showing a dividing 8- to 16-cell stage mouse embryo labelled for EB3-dTomato and H2B-GFP. These embryos were treated with PST-1P according to the described protocol and kept in dark conditions (PST-1P is inactive). These data demonstrate an almost identical temporal spindle behaviour in control and PST-1P treated embryos, and thus we can conclude that inactive PST-1P does not affect spindle or microtubule dynamics.

We would also like to direct the reviewer to Figure 3 in which an embryo had been cultured in PST-1P from 8-cell stage to blastocyst stage, and kept in the dark. This blastocyst shows no evidence of fragmentation, blebbing, irregularly-sized cells, cell death, or other morphological abnormalities. The rate of blastocyst development is a common measure used by embryo biologists to assess the developmental competence of embryos (Son, Lee et al. 2005). Therefore, we have added a new

supplementary figure (Supplementary Figure 1) to show the rates of development to blastocyst stage in control and PST-1P treated embryos.

We will also include footage of pre- and post-activation EB3-dTomato comet dynamics in the video protocol, which will complement the written protocol.

The same principle applies in fig 3 - where are the EB3 comets in this case, and so are the authors sure the drug has had no effect?

By blastocyst stage, expression of exogenous fluorescently-tagged markers becomes weaker as the cell numbers increase. This feature is not unique to EB3-dTomato or to PST treatment and is a widely acknowledged limitation of live imaging of preimplantation mouse embryos. We kindly refer back to our answer above regarding RNA degradation and dilution over the time course of preimplantation development. As a consequence, at blastocyst stage, which is 4.5 days after cRNA was microinjected at the 1-CS, the signal of the fluorescent protein will lose its intensity significantly.

The reason for Figure 3 was to demonstrate that PST-1P treated embryos can reach blastocyst stage when kept in the dark, which is a hallmark for good embryo quality as explained above. Thus, the DIC image is of more value than the EB3-dTomato labelling, which is simply a proof that is the same embryo which was originally microinjected with EB3-dTomato. This figure was not intended to demonstrate clear comets for tracking due to limited fluorophore expression in the blastocyst.

In Fig.2B, the authors claim that EB3-dTomato signal loss following 405nm laser activation is not a result of photobleaching. Although, the elapsed time between panels ii and iii it is not clearly specified, line 358 states that the image was taken immediately following 405nm laser exposure. This would imply that the depolymerization of the existing microtubules occurs momentarily. This seems very unlikely. Is there existing evidence to show that such microtubule depolymerization rate is possible?

We thank the reviewer for this comment and have added further detail to Step 3.11 to emphasise that post-activation imaging should take place as soon as possible after activation:

“3.11 Switch laser back to 561 nm and repeat acquisition as in Step 3.9 to confirm loss of EB3-dTomato comets after activation of PST-1P (Figure 1E). This acquisition should take place as soon as possible following activation.”

Practicably, this is expected to be less than 5 seconds to allow time for resetting imaging parameters and initiating the image acquisition.

In reference to the reviewer's comment on microtubule depolymerisation, Zenker et al. (Zenker et al. 2017, *Science*), demonstrated that stabilised microtubules, labelled with RFP-MAP2c, were not affected by activation of PST-1P. Here we instead show a reduction in EB3-dTomato, which labels growing microtubules. Therefore, a reduction in EB3-dTomato will occur when microtubule polymerisation is inhibited by PST-1P activation, however, this does not necessarily demonstrate microtubule depolymerisation. In summary, we show inhibition of new microtubule growth using PST-1P, and not depolymerisation of existing microtubules.

Putting together the above comments, as someone well versed in such methods in this system, I look at fig 2 and 3 and think that the chemical probably was affecting MT dynamics pre-activation, and that bleaching probably is a component of the observed effect.

Kindly referring to our answers above, we would like to highlight that although PST-1P is a robust tool to use, its application on the living preimplantation mouse embryo could increase some variability which might not to be observed in other cell systems. However, when comparing PST-1P treated and untreated embryos pre-activation as shown in Figure 2Aii and 2Bii and Supplementary Figure 2, we have assessed that there is minimal effect on microtubule growth in inactive PST-1P. Footage of a time-lapse movie showing EB3-dTomato comet dynamics will be included in the video protocol to further verify the inactivity of PST-1P prior to light activation.

We have also assessed the risk of photobleaching in untreated controls before and after mock light activation and there were no discernible differences between untreated embryos pre- and post-activation as shown in Figure 2Aii and 2Aiii.

Re Deactivation. In lines 298-299, the authors state that the spontaneous loss of EB3-dTomato signal following intentional PST-1P activation lasts between 2-20 min, after which the signal reverts to the previous state. It is unclear why controlled 514nm laser-mediated deactivation takes substantially more time (few hours, line 311).

We would like to clarify that the recommended recovery period of a few hours is to allow for restoring overall cellular function, not just microtubule growth/EB3 signal recovery. An extended recovery period following accidental PST activation will allow the cell to recover from any unintended inhibition of unknown strength and ensure an experiment under optimal conditions.

We have mentioned on line 327 that the thermal relaxation of PST-1P is gradual but we cannot give a specific time as this depends on the strength of activation, cell type, and media volume. Using a 514 nm laser to deactivate PST-1P achieves rapid recovery of the microtubules, as shown in Figure 2B iii and iv. However, to ensure reliability with downstream applications, a recovery period of a few hours will provide

sufficient time to ensure that the cell can recover its normal function. This is merely a precautionary measure to ensure best results.

We have updated line 340, to clarify this for readers.
"In this circumstance, a period of recovery for the cells is required as a precautionary measure to ensure cells can return to their pre-activation state. Ideally, this would be a minimum of a few hours, if time permits."

Re: Non-invasiveness. The authors state that the application of PST-1P has the potential for non-invasive cytoskeleton manipulations (line 41) but show no evidence that repeated UV light exposure (required to induce any lasting effects on microtubules) would not harm the embryos. Indeed, UV light has long been known to severely damage live eggs and embryos. This should be discussed.

We agree with reviewer 2 that UV light can potentially be toxic when used in excess. Thus, we wrote this protocol for the purpose of assisting users to apply the best settings to limit/avoid photo-cytotoxicity. Furthermore, to minimise the UV light exposure we also provide the user an optimised PST-1P concentration. In this context, we refer to the use of light as a non-invasive tool as it does not require physical manipulation (for example, injection or biopsy) of the embryo.

In this protocol, embryos are exposed to a 405 nm laser line at or below 10% laser power in a selected small region of interest for approximately 16 seconds. This exposure is comparatively much shorter than typically used for live-cell imaging of blue-light excited fluorophores (for example, Hoechst dye), which has been widely used in many live-cell imaging systems.

We have added additional advice to lines 446 – 449 to indicate that users of this protocol should consider the vulnerability of their samples to phototoxicity.

"To mitigate this, repeated photoactivation may be necessary, the cytotoxicity of which would need to be assessed on an individual cell-type basis as some systems may be susceptible to phototoxicity."

The use of the word "pioneered" in the context here is a bit OTT and inelegant.

We have updated line 54 to:
"Here, the application of PSTs in the 3-dimensional (3D), live preimplantation mouse embryo is set out, to disrupt the microtubule network on a subcellular level."

Reference List

Mashiko, D., Z. Ikeda, T. Yao, M. Tokoro, N. Fukunaga, Y. Asada and K. Yamagata (2020). "Chromosome segregation error during early cleavage in mouse pre-implantation embryo does not necessarily cause developmental failure after blastocyst stage." Sci Rep **10**(1): 854.

Son, W. Y., S. Y. Lee and J. H. Lim (2005). "Fertilization, cleavage and blastocyst development according to the maturation timing of oocytes in in vitro maturation cycles." Hum Reprod **20**(11): 3204-3207.

Zenker, J., M. D. White, R. M. Templin, R. G. Parton, O. Thorn-Seshold, S. Bissiere and N. Plachta (2017). "A microtubule-organizing center directing intracellular transport in the early mouse embryo." Science **357**(6354): 925-928.