Milan, 14/10/2019

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Rebuttal letter to the reviewers of the manuscripts JoVE60712

Below we provide detailed answers to the editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We have carefully checked the entire manuscript for any spelling or grammar issues.

2. Title: Please revise to avoid the use of punctuation (colon, dash, etc.) and abbreviations.
We changed the title removing the colon.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.
We have carefully checked through the manuscript for the proper use of abbreviations and symbols.

4. 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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Marienfeld, Nikon Instruments, etc.
We have removed any company names before instruments and reagents.

5. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
We have revised the protocol following editorial suggestions.

6. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE."
We have revised the protocol following editorial suggestions.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please specify all volumes and concentrations used throughout. We need these details to film. See examples below.
Following editorial suggestions, we have added more details to the protocol throughout the text.

8. 1.1: Please specify nick translation procedure. Table 2 only shows reagents and concentrations.
Reagents and concentrations are now specified in the section 1.2.

9. 1.2: What is incubated?

The subject is now specified.

10. 1.3: What probes? From which step? It is unclear. What volume of DNA Pol I, DNase I, and EDTA are added? Please specify.
Please note that all the details required are now present in the mentioned section.

11. 1.4: What happens after centrifugation, discard the supernatant?
We have added the required details in the 1.3 section.

12. 2.1.1: What does 1.5 H mean?
It means "thickness No. 1.5H". We have added the proper nomenclature in the text.

13. 2.1.3: What does "repeat other two times" mean exactly? Make two drops?
We have specified that the step 2.1.3 should be performed three times.

14. 2.2.1: Please specify the cell type and growth conditions.
As requested, we have specified the cell type and growing conditions.

15. Section 5: Please describe in the imperative tense how images are collected and analyzed.
We have specified the editor requirement within the text.

16. Figure 4: Please ensure that the panels are of the same dimensions if possible.
The panels shown are checked to be of the same dimensions.

17. Table of Materials: Please sort the materials alphabetically by material name.
Materials in the table are alphabetically sorted.

18. References: Please do not abbreviate journal titles; use full journal name.
We have used journal full name in the References.

Below we provide detailed answers to the referees' comments:

Reviewer #1:
Major Concerns:

- Paragraph 3/hybridization should include the post-hybridization washes in SSC, i.e. until step 4.4., before the blocking step which is dedicated to the FISH detection per se (paragraph 4).
We have re-organized the paragraph following reviewer suggestion.

- I understand that the Freeze-thaw steps in 20% Glycerol/PBS are for permeabilization, but is it really necessary since there is already a permeabilization step with high Triton concentration and a deproteination step with HCl or HCl/pepsin. This step is time consuming, need dry ice or liquid nitrogen, but is probably facultative (as we experienced with different mammalian cells), especially with small probes (<200 bp as described here).
We thank the reviewer for this comment. Indeed, as he noted, freeze-thaw steps are facultative and dependent on the cell type and probes sizes; therefore, we have now indicated a range from 1h to over night (O.N.) for the step of 20 % Glycerol/PBS, in a way that the reader can vary the protocol adjusting to the cell type used. However, in our experience, the freeze and thaw step in general ameliorates the result; in agreement with this, while this step is not included in protocols that propose the alcoholic scale to fix and to permeabilize cells (Chaumeil, J. 2008; Byron, M. 2013; Takizawa, T. 2008), it is always suggested when the protocol avoid alcoholic scale, like our one (Solovei, I. 2010; Cremer, M. 2008).

- If using only directly labeled probes (which often give better signal to noise ratio), the blocking/post-fix steps are unnecessary, again lightening the protocol. Kits to produce directly labeled probes by nick-translation with various Alexa fluorochromes are commercially available.
Following reviewer suggestion, we have removed the blocking and post-fixing steps for directly labelled probes, and we have better specified the different possibilities with an appropriate note in the section 1.2.

- In the representative results/discussion: may be worth to mention that "Timing of denaturation" as well as temperature of denaturation can be augmented, up to 80°C. Otherwise, to improve signal to noise ratio, temperature of hybridization can go up to 42°C.
We thank the reviewer for the suggestion, we have added a note in the section 3.5 and 3.6 to provide this information.

- In Figure 4C-D, would need to have a FISH example with optimal HCl/pepsin conditions for primary myoblasts to appreciate the importance of the pepsinization step. Unfortunately, only suboptimal conditions are shown here. Also, the same probe should be used for comparison.
As requested, we have added in Fig. 4E a DNA FISH example with optimal HCl/pepsin conditions; we have replaced the panel 4C with a new one in order to have the same probe used for panels C-D-E.

Minor Concerns:

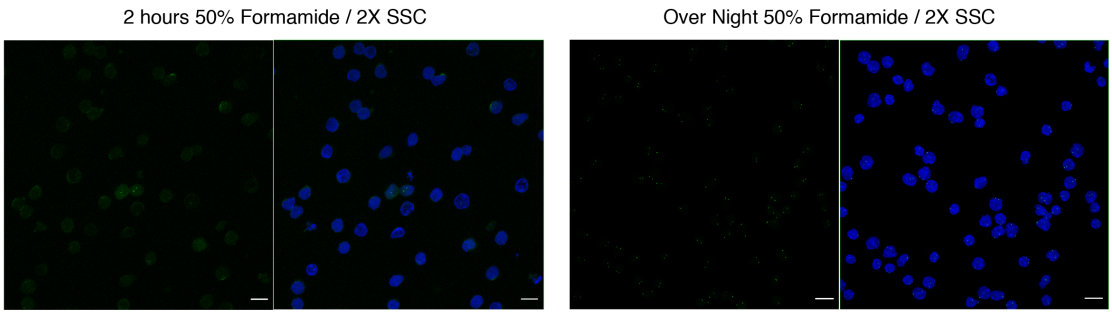
- step 2.1.3: precise for how long the Poly-Lysine drop need to remain on the glass surface. Not clear here. Alternatively, you can put the glass coverslips in a solution of 0.01% Poly-Lysine for at least 5 min.
Following the reviewer suggestion, we have specified the Poly-lysine step.

- 2.1.5: why adding few drops at the end of the fixation step. Don't think that this step is necessary.
The reviewer is right, we have removed this step from the protocol.

- 2.1.8: ON incubation in 20% Glycerol/PBS. I think 30-60 min could be sufficient.
Following reviewer recommendation, we have now indicated a range from 1h to over night (ON) for the step of 20 % Glycerol/PBS, in a way that the reader can vary the protocol adjusting to the cell type used. See also answer to Major concern point 2.

- 2.1.10: Why using DEPC water here?
This was a mistake, we have removed it.

- 2.1.11: same as above, an ON incubation seems a lot. I think 30-60 min could be sufficient. All these ON incubations render this protocol quite very long! (4 days in total, including the hybridization step).
In this particular case, although we recognized that this step could extend the time of the protocol, we have noticed that prolonged incubation in 50 % formamide/ 2× SSC seems crucial for nuclei denaturation for the subsequent hybridization step and to obtain a successful 3D DNA FISH. As the reviewer can appreciate below, 2 hours incubation is much less efficient than ON incubation with 50 % formamide/ 2× SSC. However, we have added a note at this step and at step 2.2.8, specifying that the time can be optimized and eventually reduced.



- 2.2.12: replace FA by formamide.
FA has been replaced with formamide.

- 3.1 (note): Why the denaturation of probes seems only to apply to human primary T lymphocytes. This seems an important step.
The reviewer is right; therefore, we have removed the note and extended the step as general.

- 4.6: don't understand why it is needed to block additionally when using single fluorophore, directly labeled probes.
This passage has been removed and specified, with an appropriate note in section 4.1, that for directly labelled probes the blocking is not needed.

- In the discussion: I don't think that ref . 27 (Ricci et al.) are using FISH technologies with super resolution.
As noticed by the reviewer, the citation has been corrected.

- In the discussion: "The limitation of the technology remains the small number…", Several papers have recently described sequential methods of hybridization, i.e. barcode DNA FISH (Bintu Zhuang 2018; Nir Wu 2018; Cardozo Nollmann 2019; (Nir et al. 2018; Cardozo Gizzi et al. 2019; Mateo et al. 2019)) to address genome organization in single cells. The number of loci that can be tested at a time will therefore be less and less limited.
Following reviewer suggestion, we have toned down this concept in the discussion, introducing and commenting the suggested papers.

Reviewer #2:
Major Concerns:

1. The introduction focuses on the comparison between DNA FISH and Hi-C approaches. However, this final statement is not valid: "For these reasons, although "C-technologies" are high evaluable tools to study general roles of genome folding, they need to be complemented with single cell approaches in order to dissect cellular heterogeneity". This is because single-cell HiC has already been established in other studies (Nagano et al, Nature 2013 and others) and because Hi-C approaches have already been used to complement FISH methods in previous studies (Bintu et al, Science 2018 and others). The author may extensively cut down this comparison between Hic and FISH in the introduction section.
As requested by the reviewer, we have cut and toned down the comparison between Hi-C and FISH in the introduction.

2. On the other hand, the introduction did not describe any specific feature of this particular DNA FISH protocol. The protocol appears to be a standard DNA FISH method. I do not understand how the authors have expanded on or improved the original DNA FISH protocol. This information will enable readers to understand the specific

advantage of this protocol compared to many other similar protocols. This information needs to be clarified in the introduction section in comparison with other protocols with proper citations.
We thank the reviewer for this useful suggestion. In the introduction we have now highlighted the improvements of our protocol in respect to other similar protocols.

i) In respect to other protocols (Chaumeil, J. 2008; Byron, M. 2013; Takizawa, T. 2008), we avoid the use of alcohol to maximize the preservation of the nuclear structure.
ii) In respect to protocols of 3D DNA FISH on preserved nuclei (Solovei, I. 2010; Cremer, M. 2008), we highlight the punctual adjustments in terms of permeabilization and deproteinization that we use on the basis of the cytological characteristics of the cells (e.g. different degree of nuclear compaction, cytoskeleton abundance), that in our experience can be crucial in 3D DNA FISH setting on different cell types.
iii) For data analysis, we have developed and designed a novel algorithm named NuCLεD (Nuclear Contacts Locator in 3D), described in Cortesi et al 2019 and in Gregoretti et al (accepted in Methods in Molecular Biology); in this ms we have further implemented the description of the method, providing principles for data analysis.

Minor Concerns:

1. In the abstract, ''C-technologies" is not clear. This term should be spelled out.
We have spelled out “C-technologies” in the abstract.

2. Preparation of pure DNA fragments is important to reduce the background of DNA FISH. How is DNA purified for BAC clones, plasmids, and PCR products? This information can be described.
We thank the reviewer for this comment. We have added this step in the protocol and the information regarding the kits used for DNA purification in Table 1 of reagents.

3. Why are the experimental conditions for nick translation different between BAC clones/plasmids and PCR products?
The time of incubation required for the reaction of nick translation is different between BACs, plasmids and PCR products since these templates have different length (ranging from 150 kb of a BAC to 2 kb of PCR products); thus, in our experience, to produce probes < 200 bp, DNAse I and DNA Polymerase I require different incubation times. This is now specified in the ms (see 1.3), as requested.

4. Regarding step 1.4, why are different units used for DNA measurement ("5 □L" of nick translated PCR pool vs. "100 ng" of nick translated BAC)?
The reviewer is right, we have modified the text specifying the same units of measurements.

5. Although the authors used their own protocol for nick-translation, the use of nick-translation kits can make the preparation of DNA probes much easier. Did the author try the commercial kits?
Yes, we have tried commercial kits and they worked as well as our in house nick-translation protocol. However, we would like to suggest our method as *i)* it reduces the costs of the nick translation procedure *ii)* it can be easily modified and adapted to different targets (i.e. small DNA fragments or repetitive elements), *iii)* it is very versatile for getting probes efficiently labelled directly or indirectly. We have added a note in 1.2 section to make the reader aware of the possibility to use also commercial nick-translation kits.

6. It would be informative to describe how 3D FISH data can be analyzed.
Following reviewer suggestion, we implemented this paragraph with the description of NuCLεD algorithm for data analysis. Moreover, we highlight in text that the tool is already reported in Cortesi et al 2019 and in Gregoretti et al (accepted in Methods in Molecular Biology).