**Decision letter**

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**Author response**

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Secondly, we wanted to determine how small a sub-population of very stable binding we would be able to detect in our SMT imaging. We performed Monte Carlo simulations of CTCF binding using the Gillespie algorithm and the following assumptions:

* Photobleaching is a Poisson process with a rate constant of 1/120 s.
* Non-specific binding is a Poisson process with a residence time of 1 s and 30% of all events are non-specific.
* A very stable subpopulation binds as a Poisson process with a residence time of 10 min and X % of all events are in this very stable pool.
* Specific binding is a Poisson process with a residence time of 1 min and 100% – 30% – X% of all events are specific.

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Second, although there is clearly at most a very small bias present in the practical case of our data, we also wanted to thoroughly consider the “ideal” case or thought excercise considered by the reviewer. As the reviewer suggested, we performed extensive Monte Carlo simulations following the Euler-Maruyama scheme to assess if there could be any bias coming from free molecules being over counted due to continuous re-appearance. For simplicity, we will continue with the numbers provided by the reviewer (e.g. 50% bound) and for unspecified numbers use what we find for CTCF in mESCs. Thus, we will assume the following:

* 50% of molecules are bound and 50% are in free 3D diffusion.
* The free diffusion constant is the same as for Halo-mCTCF: 2.5 m2/s.
* For simplicity, we assume the nucleus is approximately a cube with a side length of 8 m. This assumption gives us an mESC nuclear volume very similar to that calculated for an mES nucleus as an ellipsoid (Chen et al., 2014). For purposes of our calculations, a cuboid nucleus greatly simplifies our simulations. The particles have to remain within this cube.
* We assume the laser excitation beam thickness is 4 m under highly inclined and laminated optical sheet illumination which we used for single-molecule tracking. This number is based on Tokunaga et al., 2008. Thus, since the nucleus is modeled as a cube with a diameter of 8 m, half the nucleus is being illuminated. For simplicity, we model the excitation beam as a step-function with uniform photobleaching probability.
* We assume that molecules photobleach with a first-order rate constant of λ.
* We will use our experimentally determined axial detection volume of 700 nm (so just below 1/10 of the nucleus like the reviewer suggested).
* The 2D localization error is 35 nm as in our experiments. Accordingly, the 1D localization error is 25 nm.
* The time between frames, , is 4.5 ms as in our experiments.
* Since the rate of unbinding (~1 min) and the rate of re-binding (~1 min) for CTCF is negligible at a 4.5 ms frame rate, we assume molecules never shift between the free and bound states (in practice, that they photobleach before they can shift).