Online supplemental material

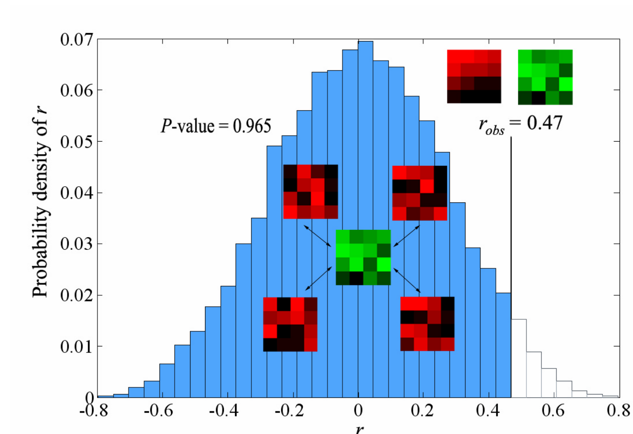

FIGURE 1S The true co-localization significance test is illustrated on a 4x4 2D image where true co-localization exists. The initial red and green channels are shown on top right in graph. The green image is the same as the red image with noise added to it to lower the amount of co-localization, leading to an overall correlation of 0.47 (i.e. $r_{obs} = 0.47$) and so the co-localization no longer being obvious by visual inspection. The probability distribution of random co-localization was obtained by computing r after repetitively scrambling the pixel positions in the red image (4 of the 200 scrambled images are shown around the center). The probability to have true co-localization is the area (shown in blue) under the probability density curve for r less than 0.47, which equals to 96.5% (*P*-value).

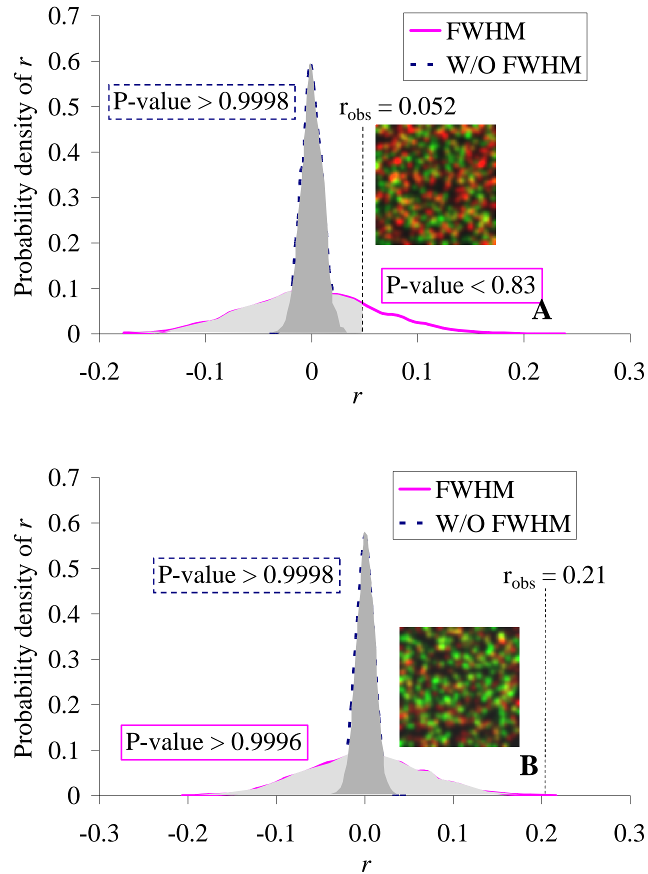

FIGURE 2S These figures illustrate the importance of taking into account the spatial correlation of neighboring pixel intensities caused by the PSF for an accurate co-localization significance test. The panels A and B show the correlation distributions of an uncorrelated (r_{obs} = 0.052) and a correlated (r_{obs} = 0.21) red-green image, respectively (based on 5000 pixel or FWHM-block randomizations). The corresponding images are shown below each graph. Images in panel A were generated by randomly seeding 5% of the image with positive intensity pixels (intensity between 0 and 255). Then each channel was blurred with a Gaussian filter with sigma equal to 2. The autocorrelation returned a value of 6 for the FWHM in both vertical and horizontal directions in both channels. Using the distribution of *r* from scrambling individual pixels wrongly concluded the presence of true co-localization (dashed distribution, blue-shaded area with P-value of 99.98%). On the other hand, when randomization was done on 6x6 pixel blocks (solid distribution, pink-shaded area), no true co-localization was detected (P-value of 83%). In panel B, images where generated in a similar manner to panel A, except that 20% of the seeds were identically located in both channels to serve as true co-localization. In this case, randomizing on a pixel basis or on a block basis both concluded there was true co-localization.

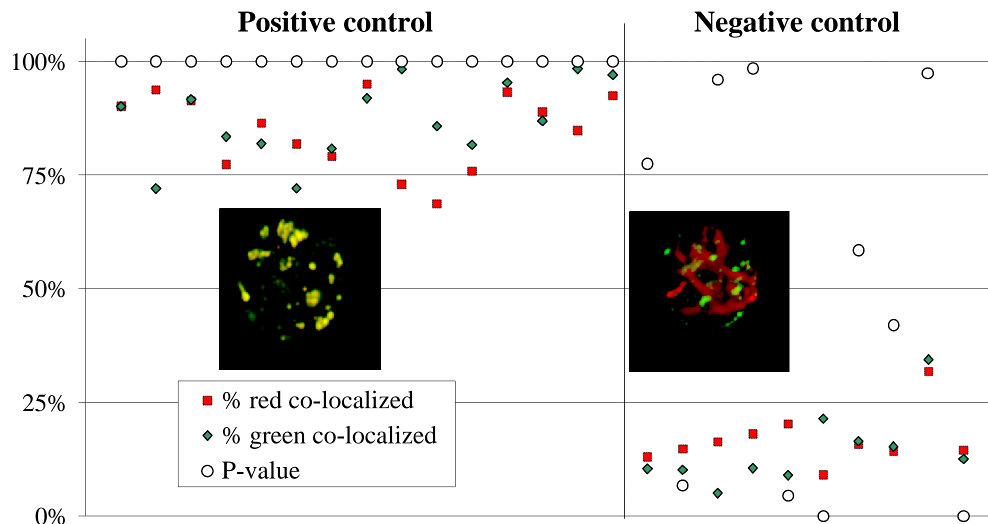

FIGURE 3S. Biological control test. Positive controls were cells stained with a primary antibody against granzyme A, and two different secondary antibodies. Negative controls were cells stained against two proteins that localize in different regions of the cells, the mitochondria and the lysosome. Each set of three points at the same vertical position on the graph represents a different cell. For the positive control, the probability of non-random co-localization (*P*-value) was always greater than 99.5% (200 randomizations used) and the amount of co-localization was around 85%. On the other hand, for the negative controls, co-localization was on average around 15% and *P*-values were much less than the 95% significance level for the most part. Therefore measurements for this group were the result of random co-localization *(Costes, S., Cho, E., Catalfamo, M., Karpova, T., McNally, J., Henkart, P. & Lockett, S. (2002) Proceedings Microsc. Microanal.* ***8 (Suppl. 2)****).*

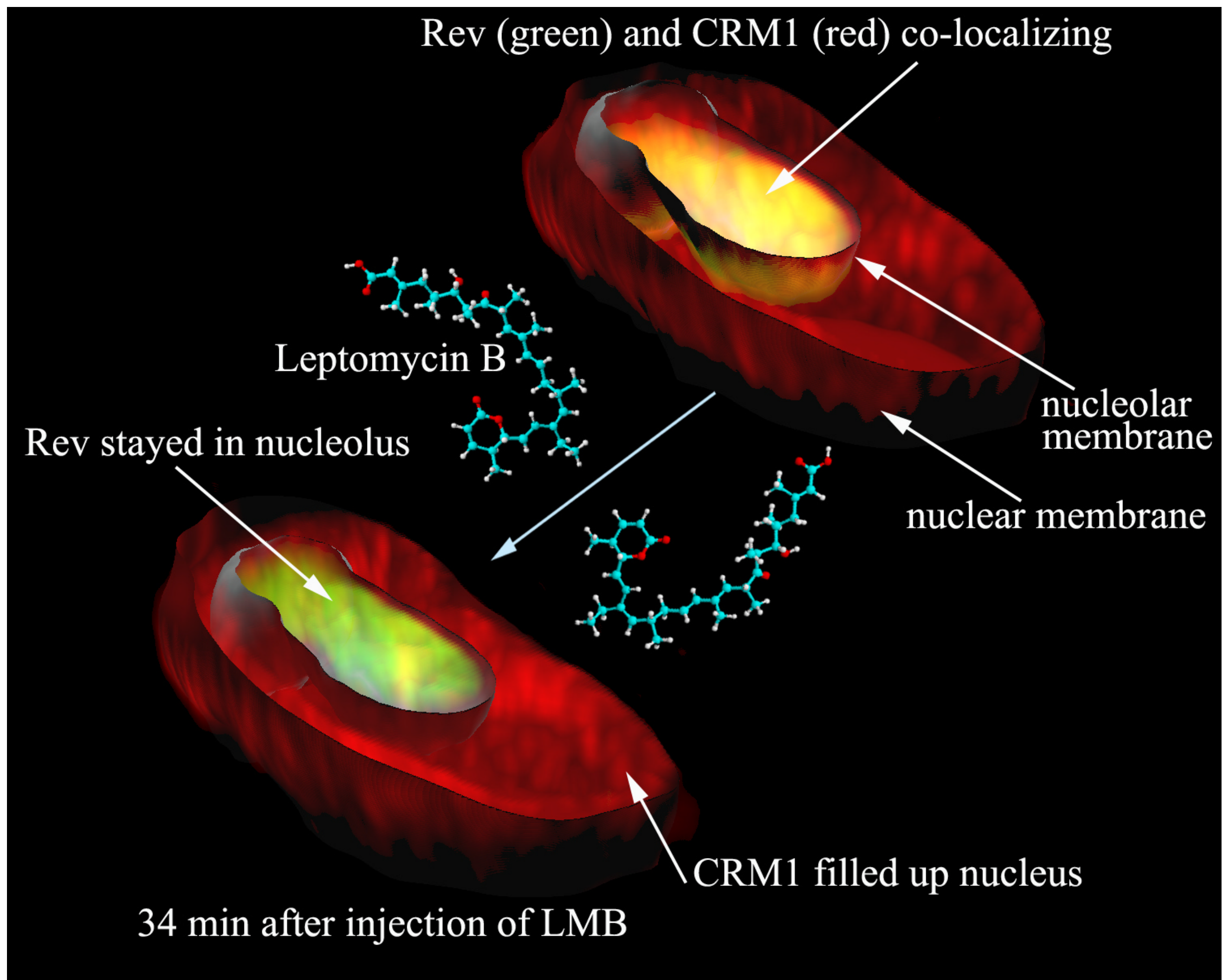

FIGURE 4S. Summary picture of the biological experiment. 3D confocal images of a live HeLa cell were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY) using 30 slices in the Z direction and 3D images were displayed along with surface segmentations with Bitplane Surpass software (Zurich, Switzerland). HIV-1 Rev protein is shown in green (CFP) and the export receptor CRM1 shown in red (YFP). Segmentations of the nuclear membrane and the nucleolus are also shown as red and gray surfaces. Two sections of the nucleus of the cell are shown before and 34 min after injection of Leptomycin B (LMB). One can see the delocalization of CRM1 from the nucleolus to the full nucleus. (THIS FIGURE COULD BE CONSIDERED FOR COVER ART SUBMISSION)