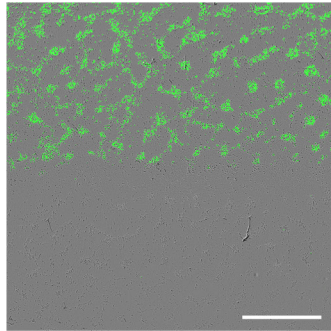
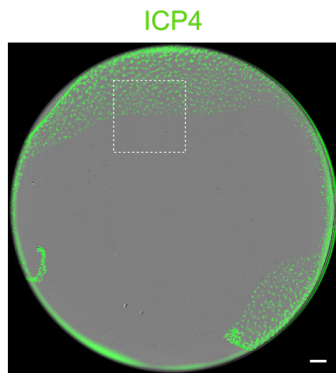


(a)



(b)

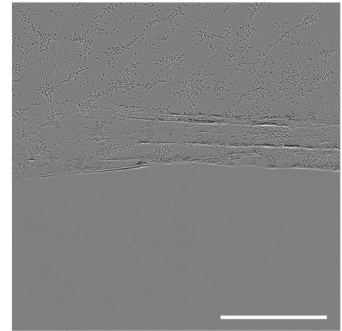


Fig. S1.

Neurone layers are prone to drying out and folding. (a) i3Neurons in a 24-well plate were infected at MOI 5 for 1 h in an inoculation volume of 200 μ L before three PBS washes. 16 hpi cells were fixed, stained for ICP4 and imaged via whole-well scanning in an Incucyte SX5. The presence of fluorescence at the edges of the well was an indication of extensive cell death, due to the centre of the well drying out during virus inoculation. (b) Phase contrast image of i3Neurone layer peeling because of pipetting down the wall of the well. Scale bars 800 μ m.

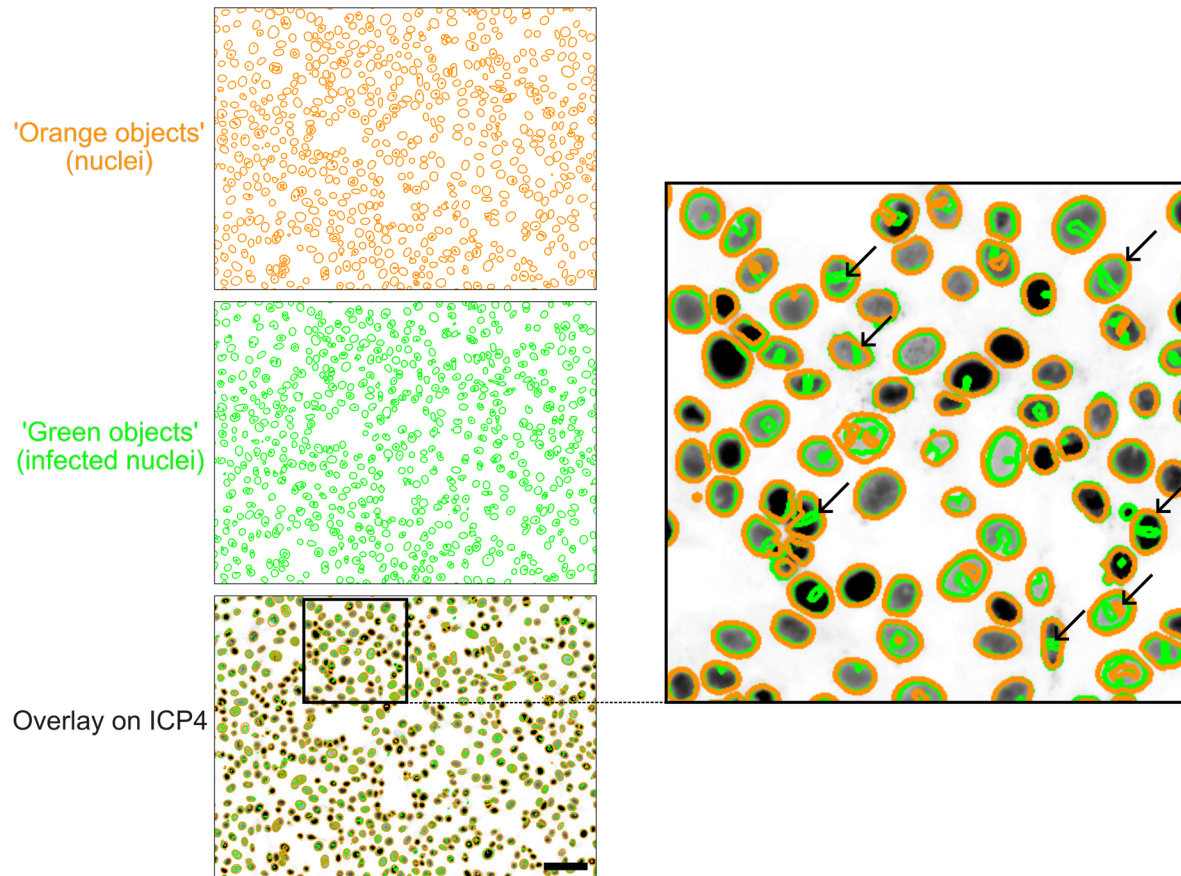


Fig. S2.

Incucyte SX5 'basic analyzer' software double-counts infected Vero cells. Arrows indicate incidences where there are two distinct objects for ICP4 signal (green) within a single nucleus (orange), which inflates the number of infected nuclei. Scale bar 100 μm .

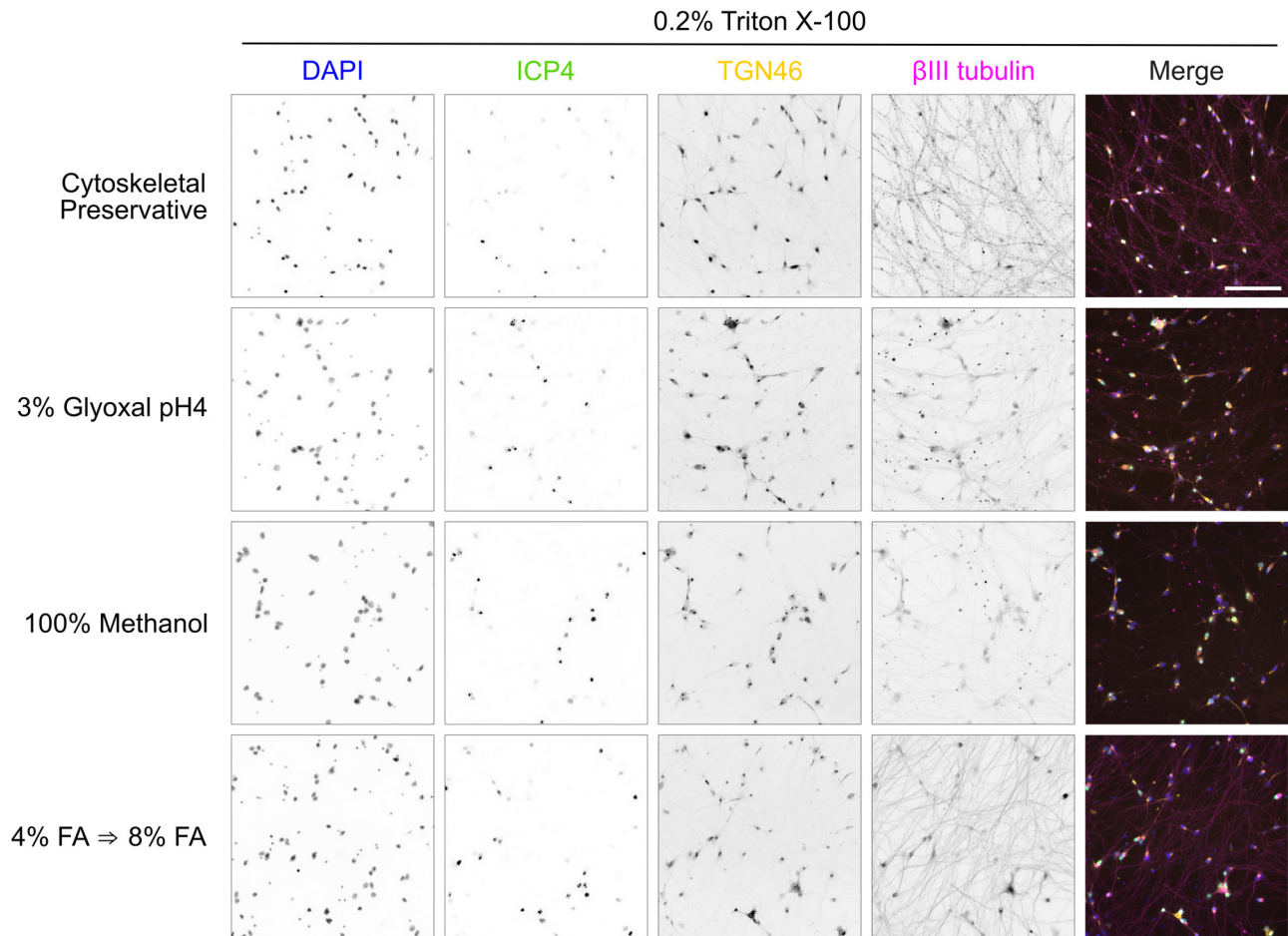


Fig. S3.

Exploration of different fixation protocols, showing that stepwise formaldehyde (FA) fixation gives the best preservation of neurone morphology and subcellular structures. i3Neurones were infected with HSV-1 (MOI 3) and fixed 16 hpi using the four listed fixatives, as detailed in the Methods. Fixed neurones were permeabilised with 0.2% Triton X-100 in PBS and immunostained for ICP4 (green), TGN46 (yellow) and β III tubulin (magenta). During coverslip mounting the cells were also stained with DAPI (blue). Scale bar 100 μ m.

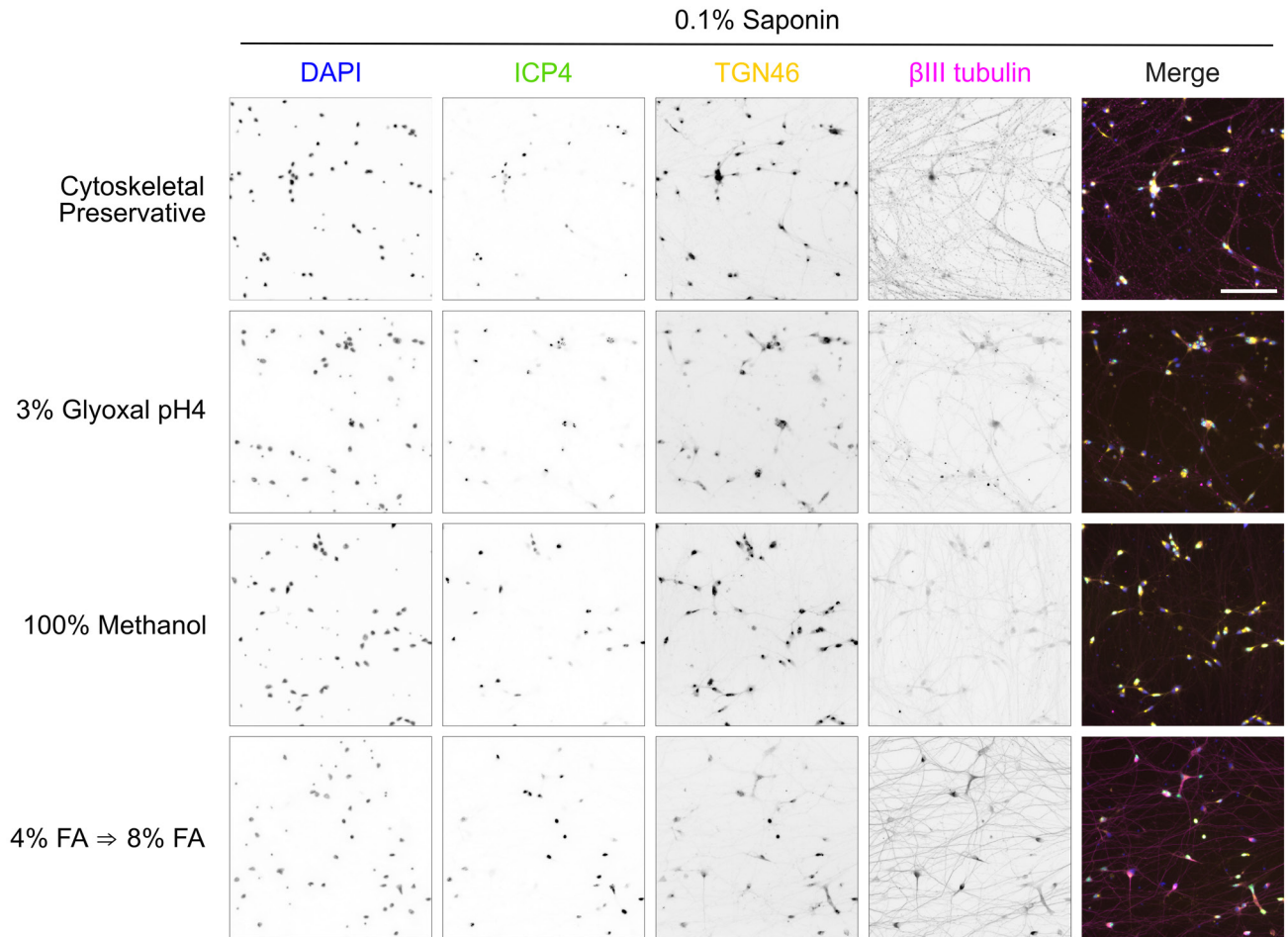


Fig. S4.

Exploration of different fixation protocols, showing that stepwise formaldehyde (FA) fixation gives the best preservation of neurone morphology and subcellular structures. i3Neurones were infected with HSV-1 (MOI 3) and fixed 16 hpi using the four listed fixatives, as detailed in the Methods. Fixed neurones were permeabilised with 0.1% saponin in PBS and immunostained for ICP4 (green), TGN46 (yellow) and β III tubulin (magenta). During coverslip mounting the cells were also stained with DAPI (blue). Scale bar 100 μ m.

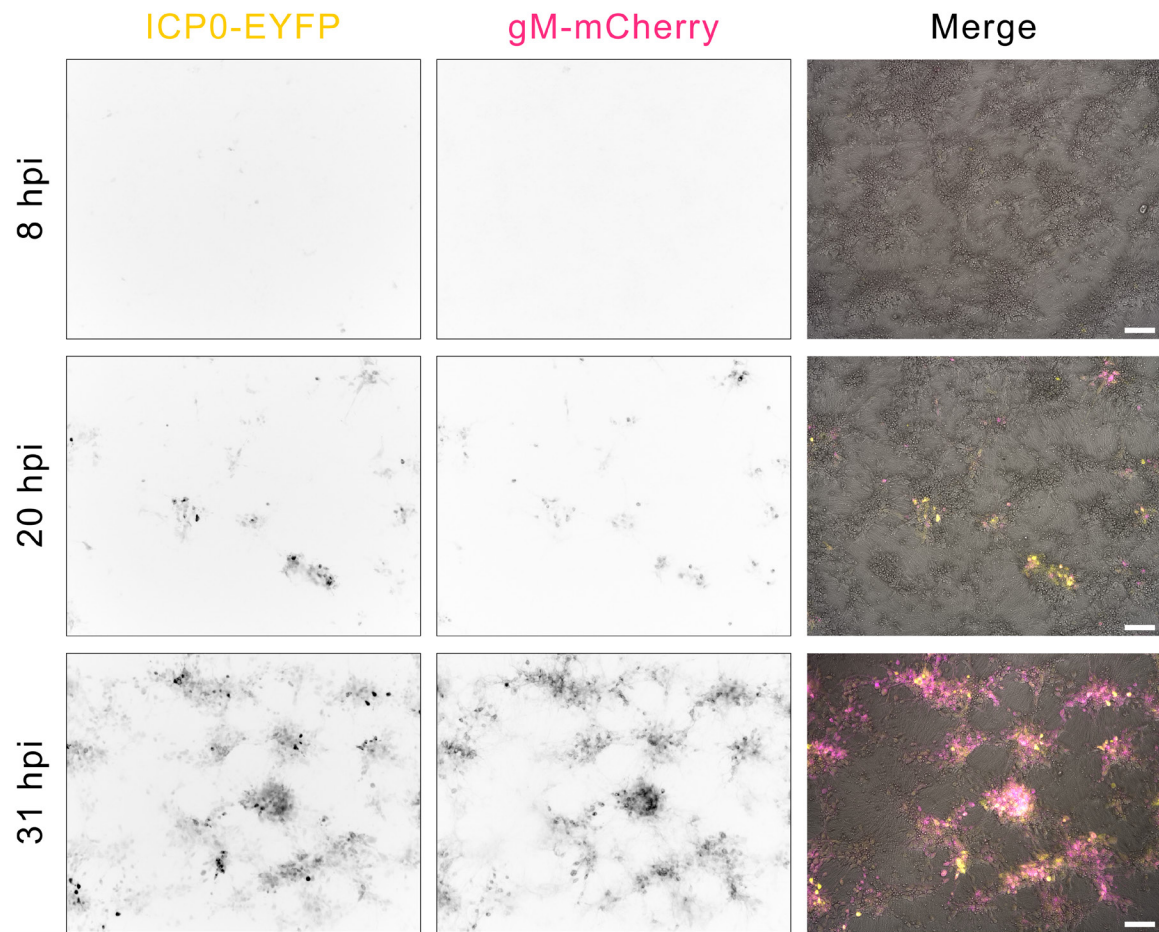


Fig. S5.

Expression of ICP0-EYFP is visible when imaging i3Neurones infected with timestamp HSV-1 on a widefield fluorescence microscope with optimised filters. i3Neurones were infected at MOI 0.1 timestamp viruses, or mock-infected, and live cells were imaged at 8, 20, or 31 hpi using an EVOS FL Auto 2 widefield microscope with 10× objective plus YFP and Texas Red EVOS LED cubes, in addition to phase contrast. ICP0-EYFP signal is evident in a subset of cells at 8 hpi and more prominent at 20 hpi, becoming more widely distributed at 20 and 31 hpi as the infection spreads. Scale bar 100 μ m.

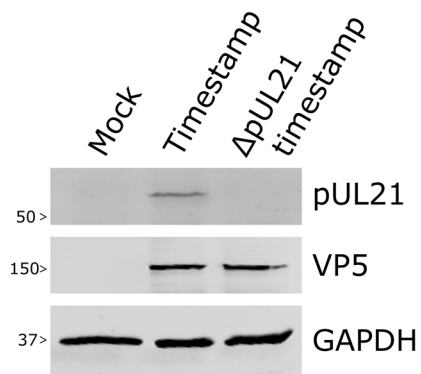


Fig. S6.

Timestamp Δ pUL21 HSV-1 lacks expression of viral protein pUL21. U2OS cells were infected at MOI 5 with parental or Δ pUL21 timestamp viruses, or mock-infected, and lysates harvested at 16 hpi. Lysates were immunoblotted for viral proteins pUL21 and VP5 plus the cellular protein GAPDH.