

Figure S1

Figure S1: Calculation of false negative error for ICCE. Data from 23 groups of 1000 HEK293T cells were manually evaluated for presence of speck-like structures in speck negative population (Spot count=0). Representative images of single cells (n=126; top). Mean frequency of cells with speck-like structures is shown \pm SEM (bottom).

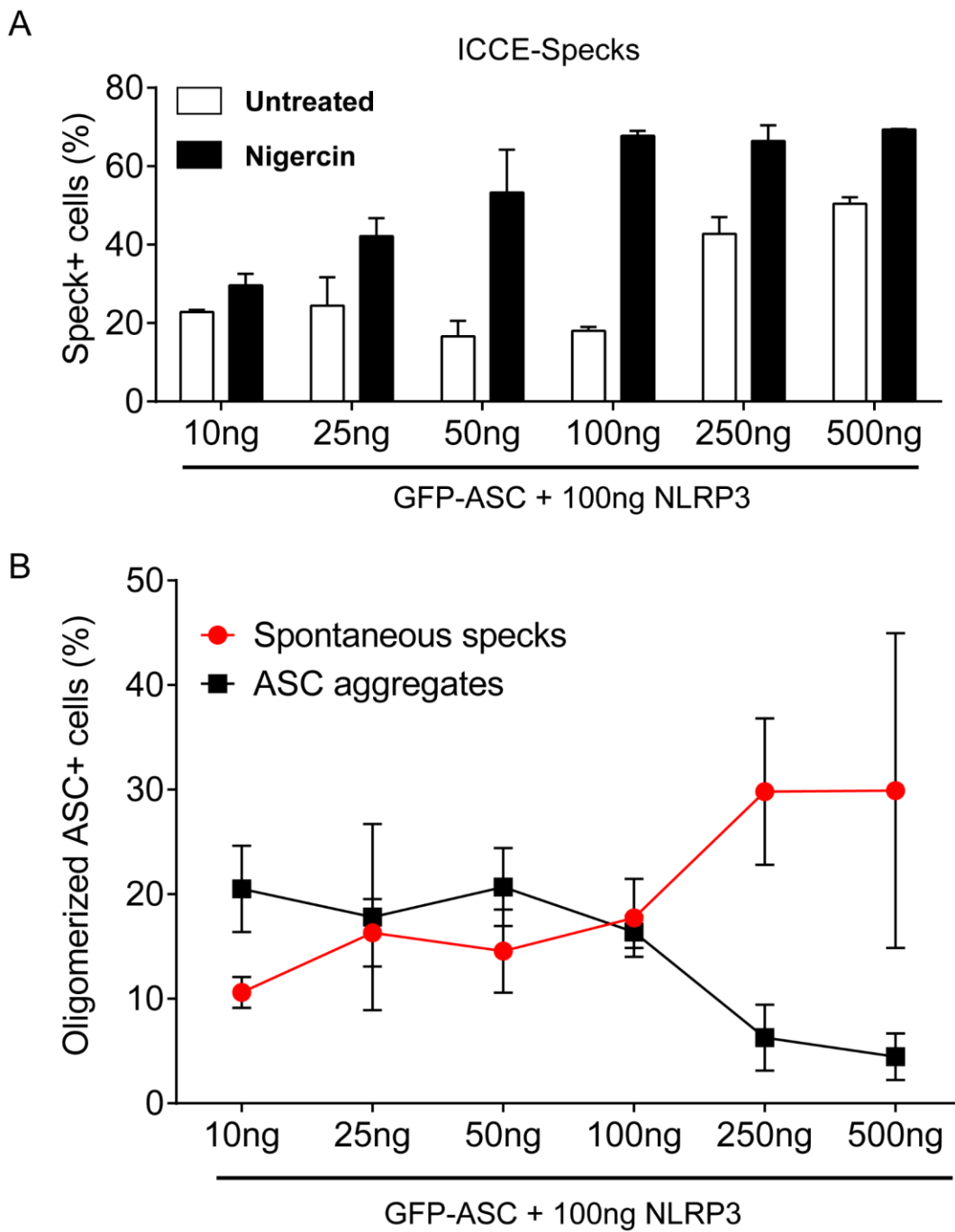
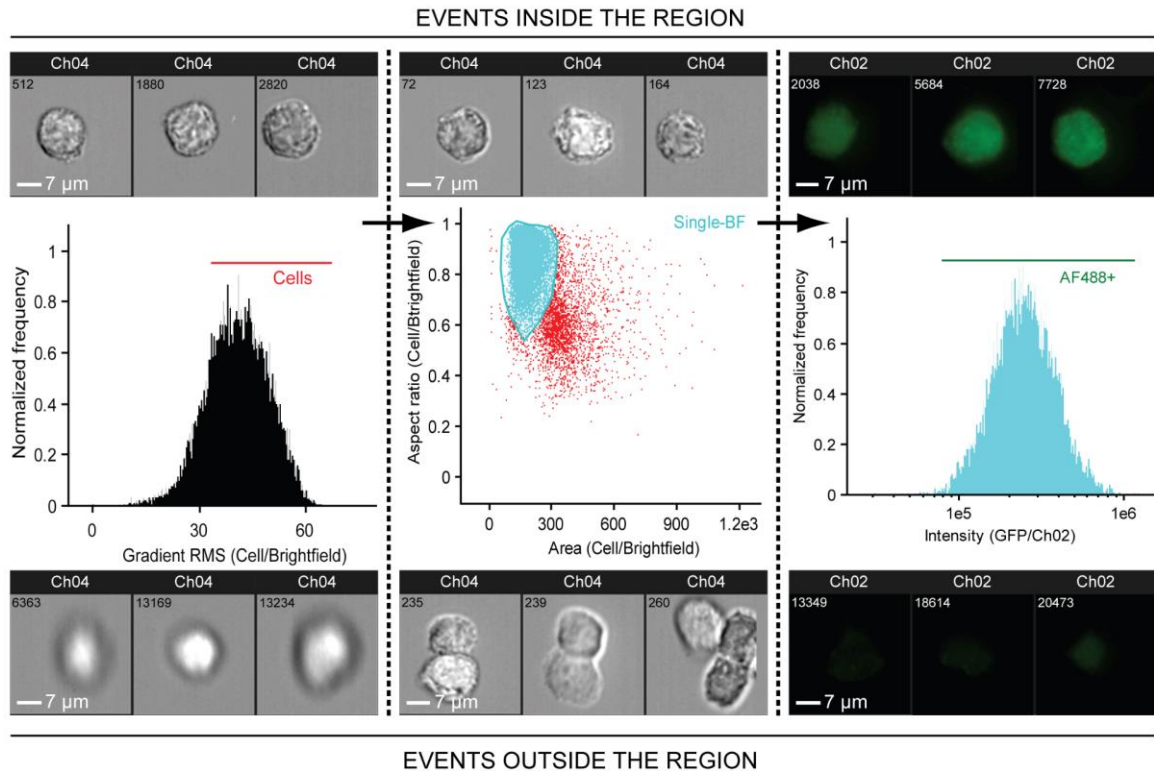


Figure S2

Figure S2: Correlation of increasing ASC concentration with spontaneous ASC speck formation and random ASC aggregates. HEK293T cells were transfected with 100ng NLRP3 and the indicated amounts of GFP-ASC and then stimulated with vehicle or 5 μ M nigericin. **(A)** Frequency of speck-positive cells (ICCE; ASC mask 14; spot count=1). **(B)** Sample described in A was analyzed for the presence of ASC aggregates (ICCE; ASC mask 8; black boxes) plotted with spontaneous specks (untreated from A; red circles). Mean \pm SEM (n=2)

A



B

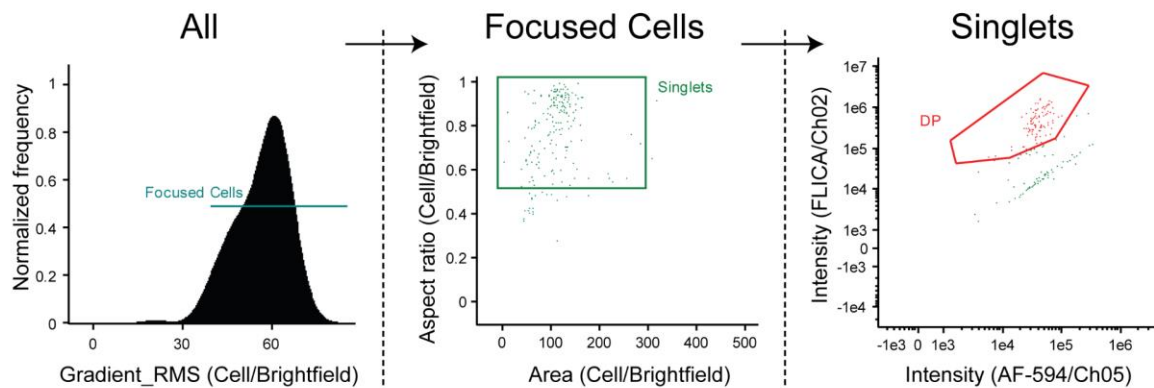


Figure S3

Figure S3: Gating strategy to identify ASC positive cells in THP1 and primary human monocytes. A. For THP-1: From left to right: histogram of Gradient_RMS of Focused cell mask with a region set to return focused cells (cells; red), dotplot of Area vs. the Aspect Ratio of the Focused cell mask with a region set to exclude non-single cell events (singlets; cyan), histogram showing intensity of Alexa Fluor 488 staining with the region set to exclude dim events (AF488+; green). **B.** For primary human monocytes: From left to right: histogram of Gradient_RMS of Focused cell mask with a region set to return focused cells (cells; blue), dotplot of Area vs. the Aspect Ratio of the Focused cell mask with a region set to exclude non-single cell events (singlets; green), dotplot showing intensity of FAM-FLICA vs Alexa Fluor 594 staining with the region set to select cells positive for ASC staining and active caspase-1 (DP; red).

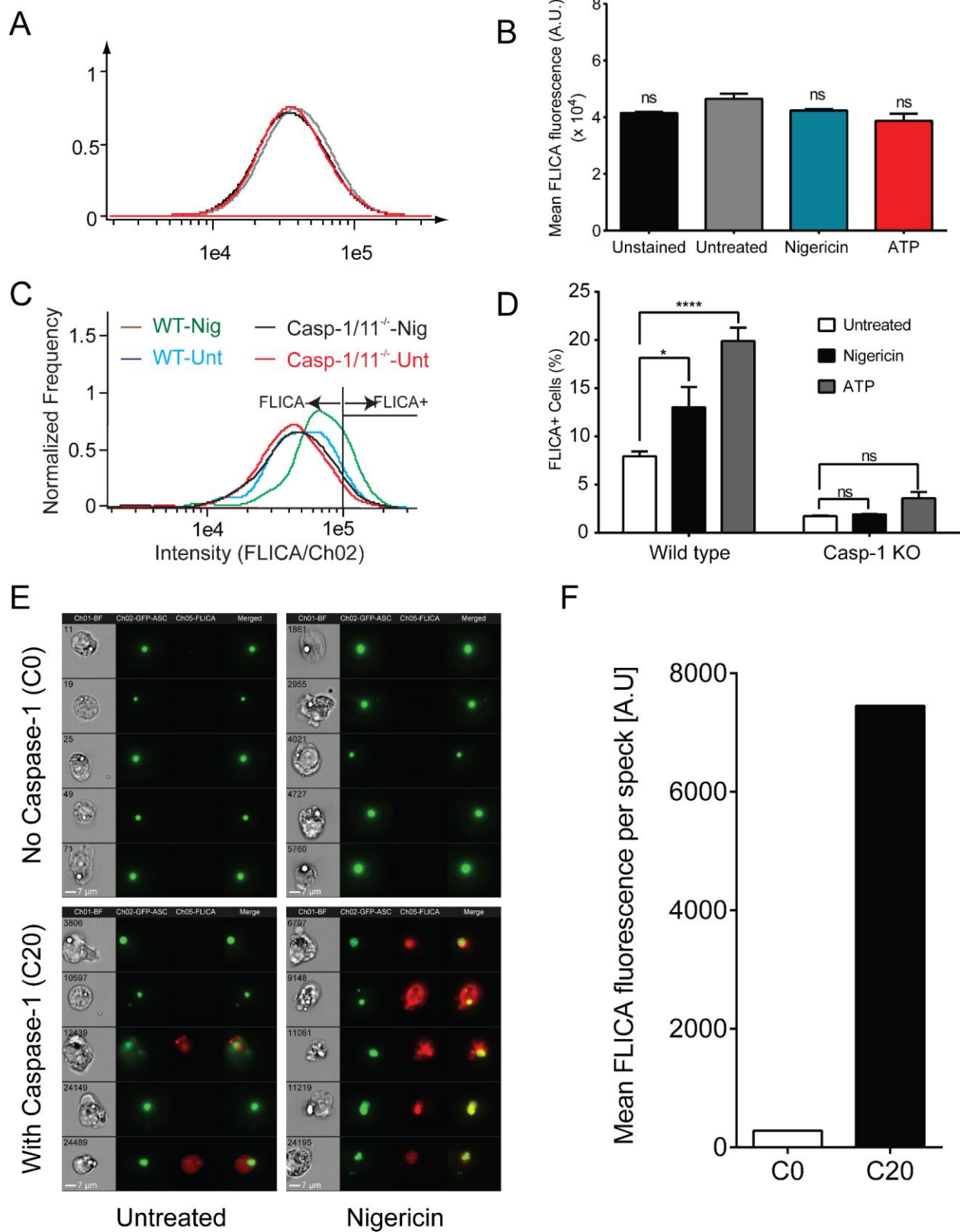


Figure S4

Figure S4: Evaluating the specificity of FLICA staining. (A) Normalized frequency and FLICA mean fluorescence intensity (MFI; intensity) of immortalized BMDMs (Casp-1/11^{-/-}) primed with 100ng/ml LPS for 3-4 hrs and stimulated without or with ATP or nigericin for 30 min. (B) Quantitation of the MFI values from (A). Mean \pm SEM, **** $p < 0.001$, One-way ANOVA followed by Tukey's multiple comparison test. (C) Normalized frequency and FLICA mean fluorescence intensity (MFI; intensity) of immortalized BMDMs (WT and Casp-1/11^{-/-}) primed with 100ng/ml LPS for 3-4 hrs and stimulated without or with nigericin for 30 min. (D) Frequency of FLICA-positive cells from the data in A. Mean \pm SEM (n=2). (E) Representative images of ASC specks showing coincident FLICA staining in cells transfected without/with caspase-1 and treated with nigericin or vehicle. (F) FLICA intensity at ASC specks. Cells with specks were counted and FLICA MFI determined without threshold application. The calculated average FLICA intensity of cells with detectable FLICA is shown.