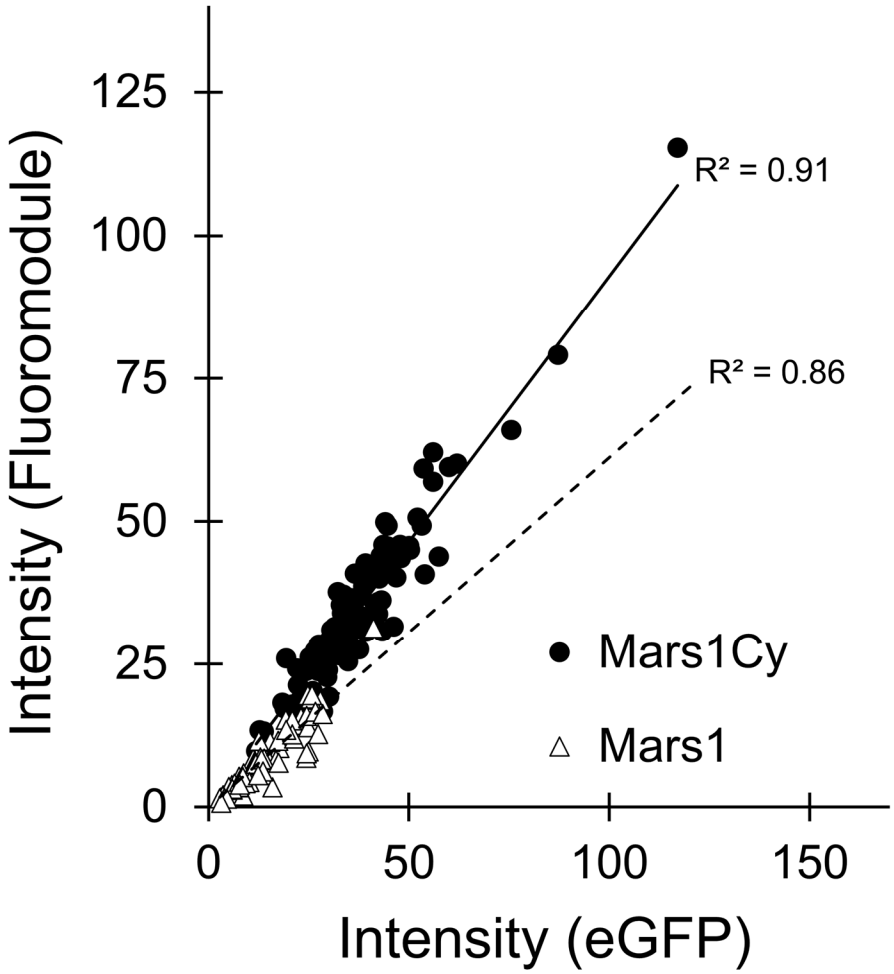
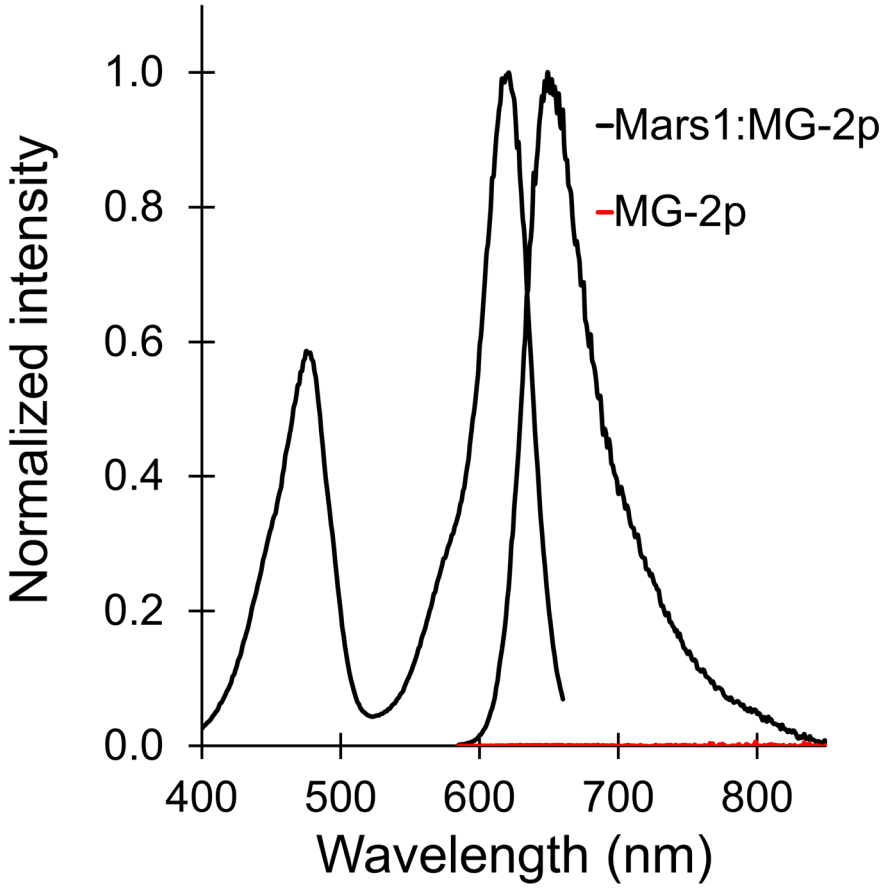
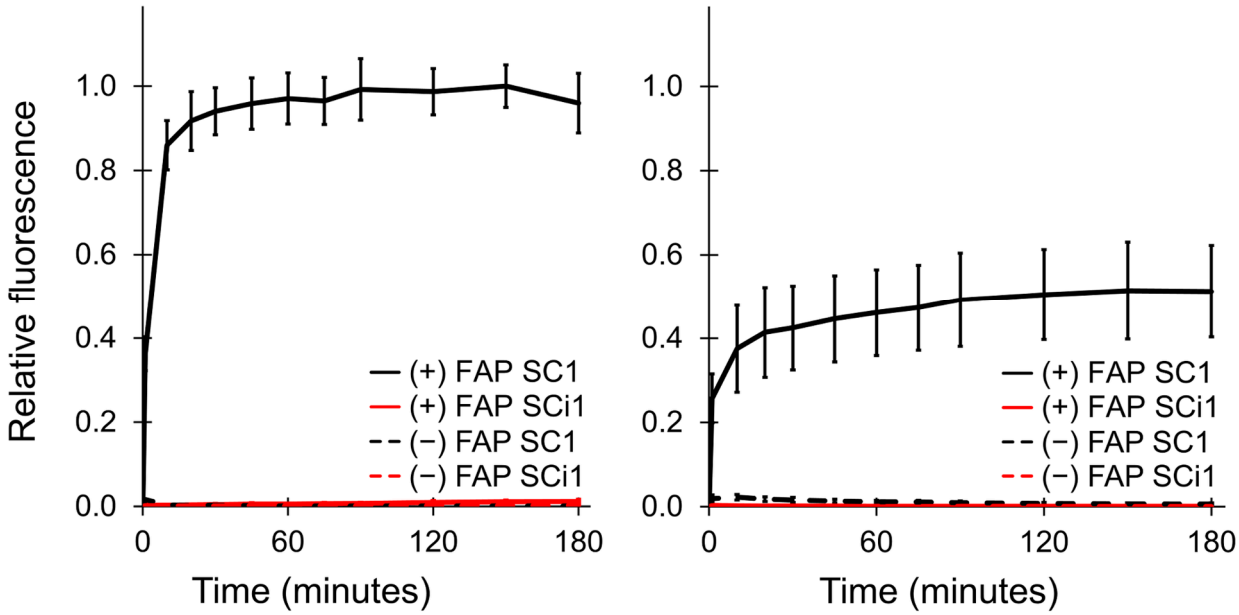
Supplemental Figures



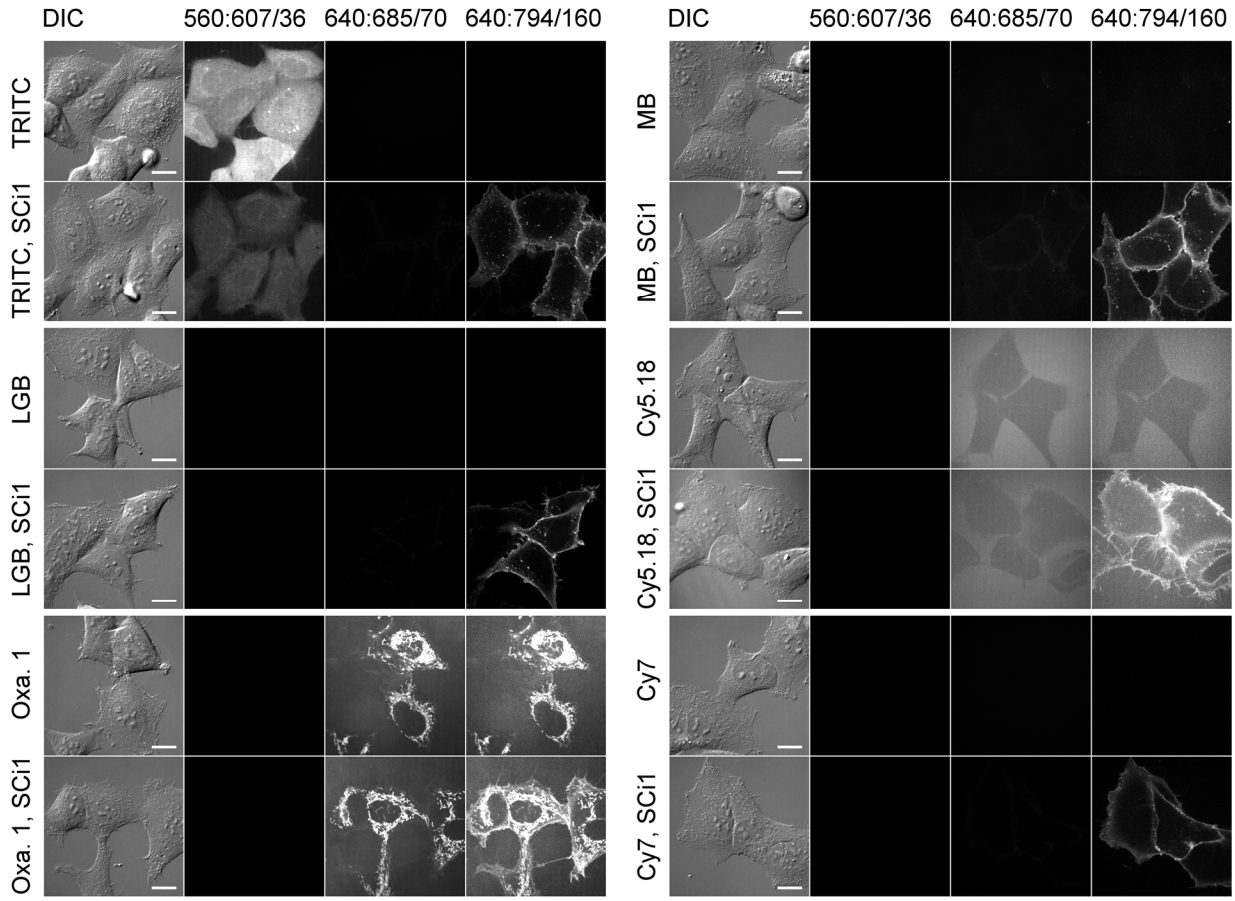
Supplemental Figure 1. Relative intensities of Mars1-eGFP and Mars1Cy-eGFP fusion proteins expressed in the cytoplasm of HEK293 cells, as measured via confocal microscopy under identical optical conditions. Individual data points represent the mean fluorescence intensity from eGFP (488:525/50) and each fluoromodule (640:794/160) fluorescence channels from single cells in standard growth media (DMEM + 10% FBS) supplemented with 200 nM SC1.



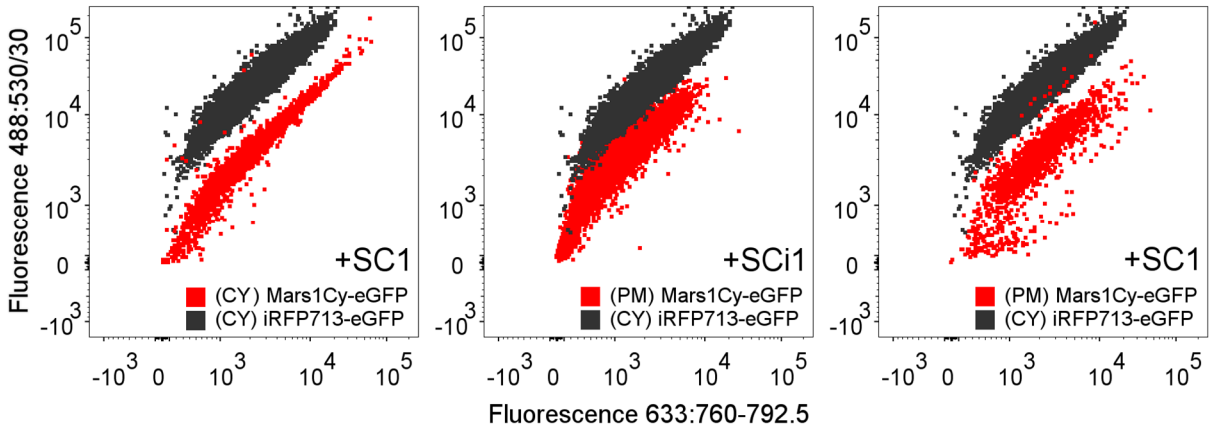
Supplemental Figure 2. Excitation and emission spectra of Mars1:MG-2p and emission spectrum of free MG-2p in PBS, pH 7.4. Mars1Cy:MG-2p and corresponding fluoromodules formed with MG-ester, a membrane-permeant derivative of MG-2p, are highly similar and omitted for clarity. Excitation peaks of the fluoromodule occur at 478 and 621 nm, enabling use with 488 and 633 nm laser lines. The wavelength of maximum emission lies at 649 nm.



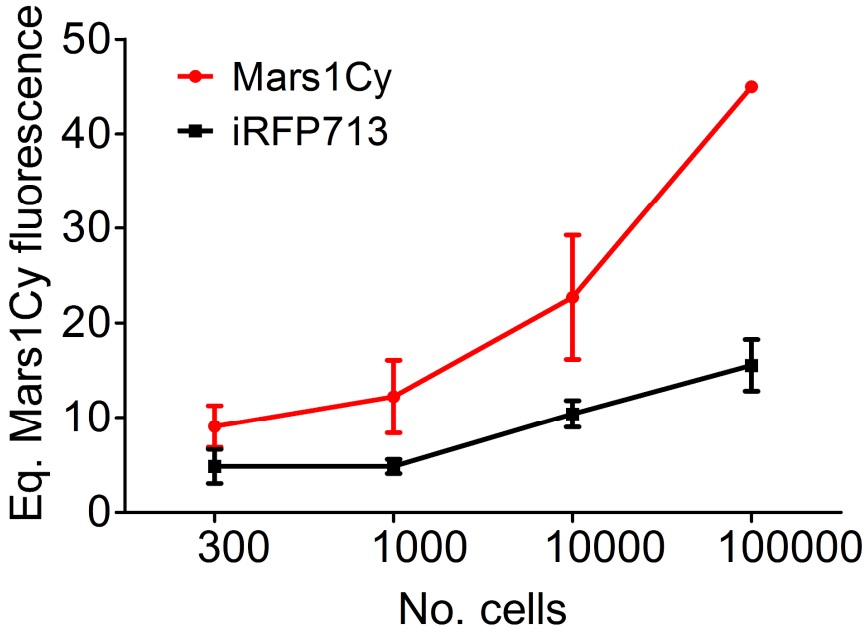
Supplemental Figure 3. SC1 and SCi1 exhibit differences in membrane permeability and the observed trends remain consistent at different temperatures. SC1 crosses cell membranes to achieve activation in cells expressing Mars1Cy-eGFP maintained at (**A**) 20 °C, and (**B**) on ice, while SCi1 remains excluded. HEK293 cells that do not express Mars1Cy do not display fluorescence activation in the presence of either fluorogen at 200 nM. Points indicate the median population MESF (calculated from relative fluorescence to a series of Cy5-decorated beads, average of three measurements collected on different days) expressed as fraction of the highest value; bars represent the standard deviation across measurements.



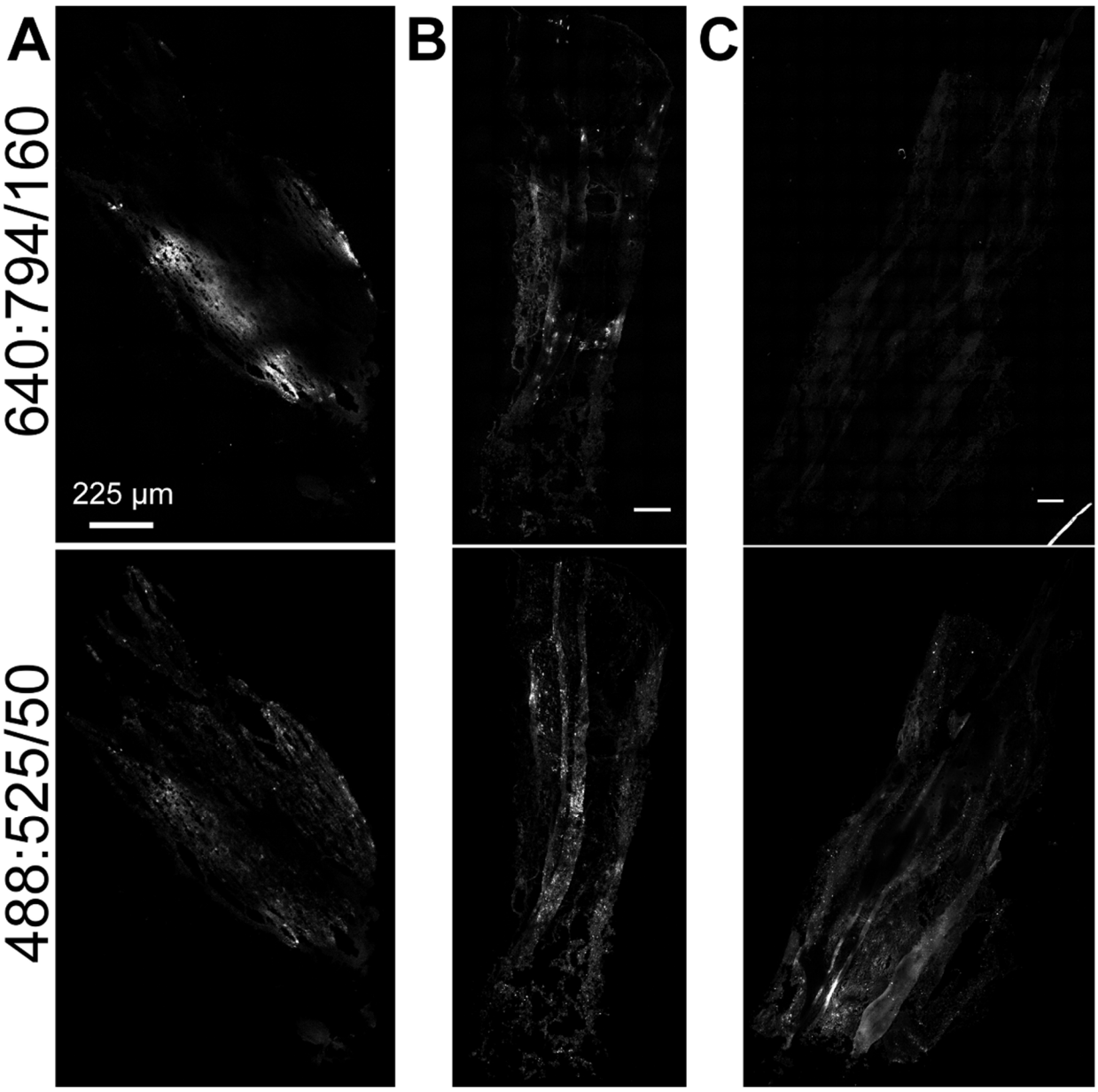
Supplemental Figure 4. Mars1 demonstrates no binding to TRITC, Lissamine Green B (LGB), Oxazine 1 (Oxa. 1), methylene blue (MB), Cy5.18, or Cy7 at 500 nM. Scale bars: 16 μm. HEK293 cells that express Mars1 on the plasma membrane were incubated with each of the six listed dyes for 60 minutes. Fluorescence under various excitation and emission conditions was acquired, in addition to bright field images. Each sample was imaged again after addition of 500 nM SCi1 without washing. The top (dye only) and bottom (dye + SCi1) sets of images for each dye display different fields of view. None of the dyes assessed, which include representatives of rhodamines, oxazines, cyanines, a phenothiazine, and a bulky triarylmethane, demonstrates signal accumulation at the plasma membrane, where Mars1 is expressed. TRITC and Oxazine 1 produce significant intracellular signal from the cytoplasm and mitochondria, respectively.



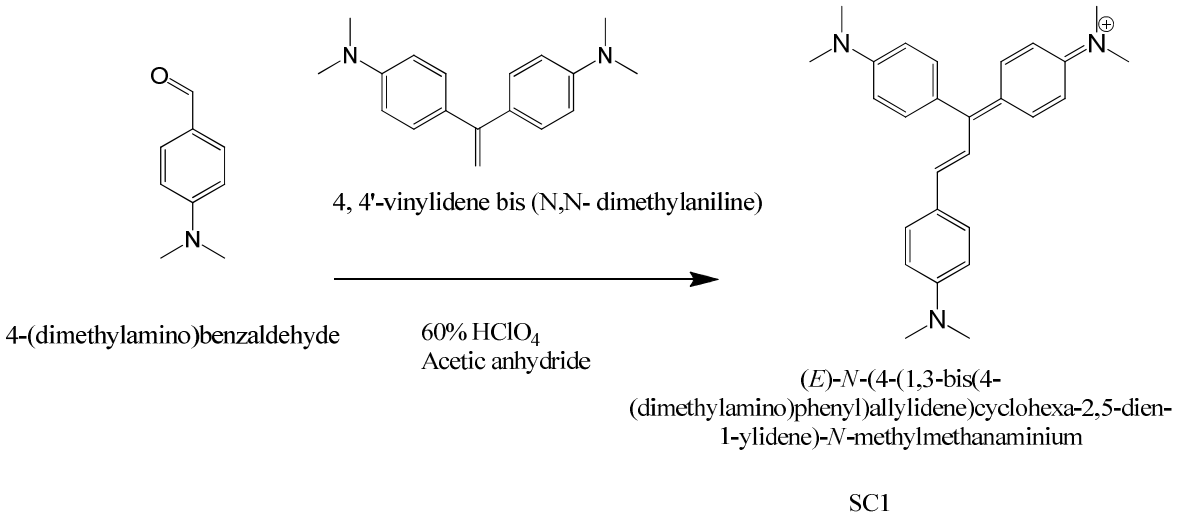
Supplemental Figure 5. HCT116 cell lines used for tumor generation and subcutaneous injections express overall greater amounts of cytosolic (CY) iRFP713-eGFP than cytosolic Mars1Cy-eGFP and plasma membrane-directed (PM) Mars1Cy, while the fluoromodule yields greater far red fluorescence per unit eGFP activity. Calibration beads bearing known quantities of Cy5 (Bangs Laboratories Inc., Fishers, IN) were used to estimate the approximate median fluorophores per cell at 2.3×10^7 (cytosolic iRFP713, 18158 events), 8.3×10^6 (cytosolic Mars1Cy, 5293 events), and 5.7×10^6 (plasma membrane Mars1Cy, 8692 events), after adjusting for each label's molar absorptivity at 633 nm, quantum yield, and fraction of fluorescence transmitted by the optical path of the flow cytometer.



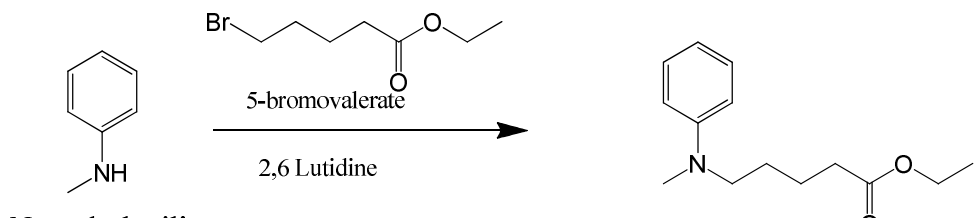
Supplemental Figure 6. Data from Figure 4B was quantified in terms of Mars1Cy fluorescence to allow for a relative measurement of signal intensities detected from each injection site. Points denote means, bars SEM. We observe a similar trend to that displayed in Figure 4B.



Supplemental Figure 7. Assessment of in vivo-labeled tumors by fluorescence microscopy after extraction, sectioning, and fixation. Scale bars displayed in the 640:794/160 channels represent 225 µm. (**A**) Tumor slice composed of HCT116 cells that express Mars1Cy-eGFP on the plasma membrane surface. Membrane-impermeant fluorogen SCi1 was administered via IP injection into the host animal prior to extraction. (**B**) Tumor slice composed of HCT116 cells identical to those in a; labeling was performed via IP injection of membrane permeant SC1. (**C**) Tumor slice composed of HCT116 cells that express Mars1Cy-eGFP in the cytoplasm; labeling was performed as in **B**. Images are composed of tiled fields of view assembled with the Grid/Collection stitching plugin for ImageJ (53).



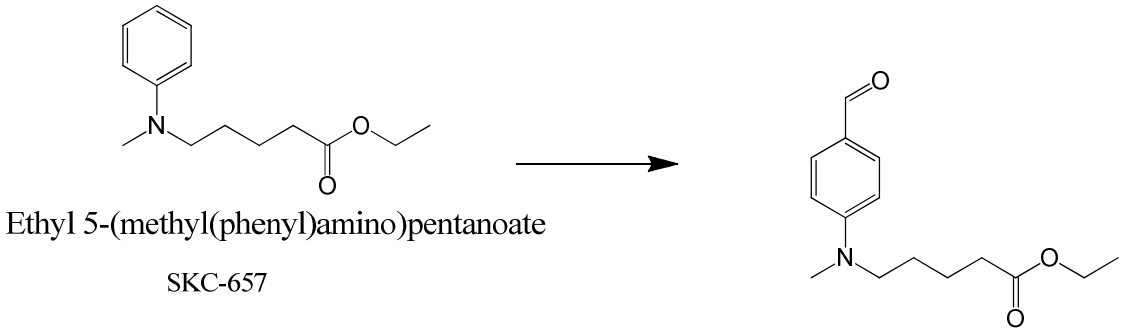
Supplemental Figure 8. Reaction scheme for synthesis of SC1.



N-methylaniline

Ethyl 5-(methyl(phenyl)amino)pentanoate

SKC-657

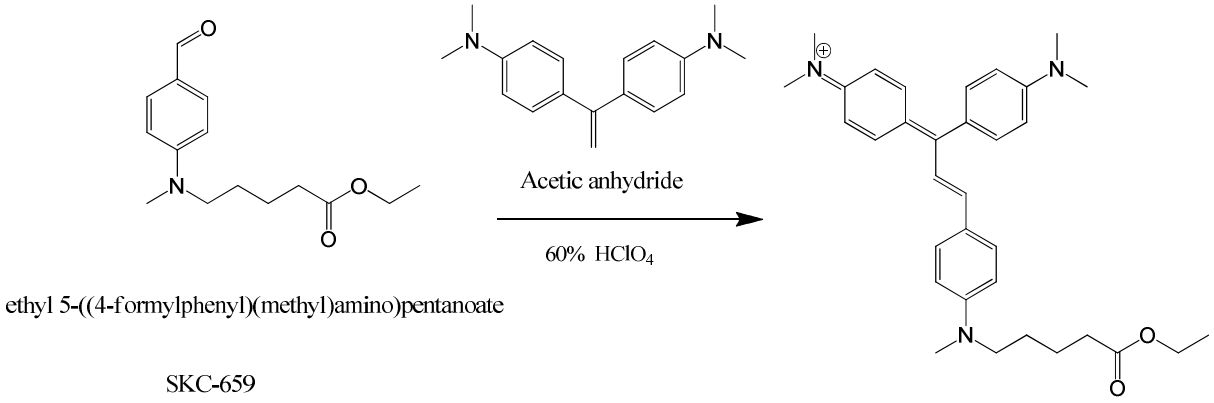


Ethyl 5-(methyl(phenyl)amino)pentanoate

SKC-657

Ethyl 5-((4-formylphenyl)(methyl)amino)pentanoate

SKC-659



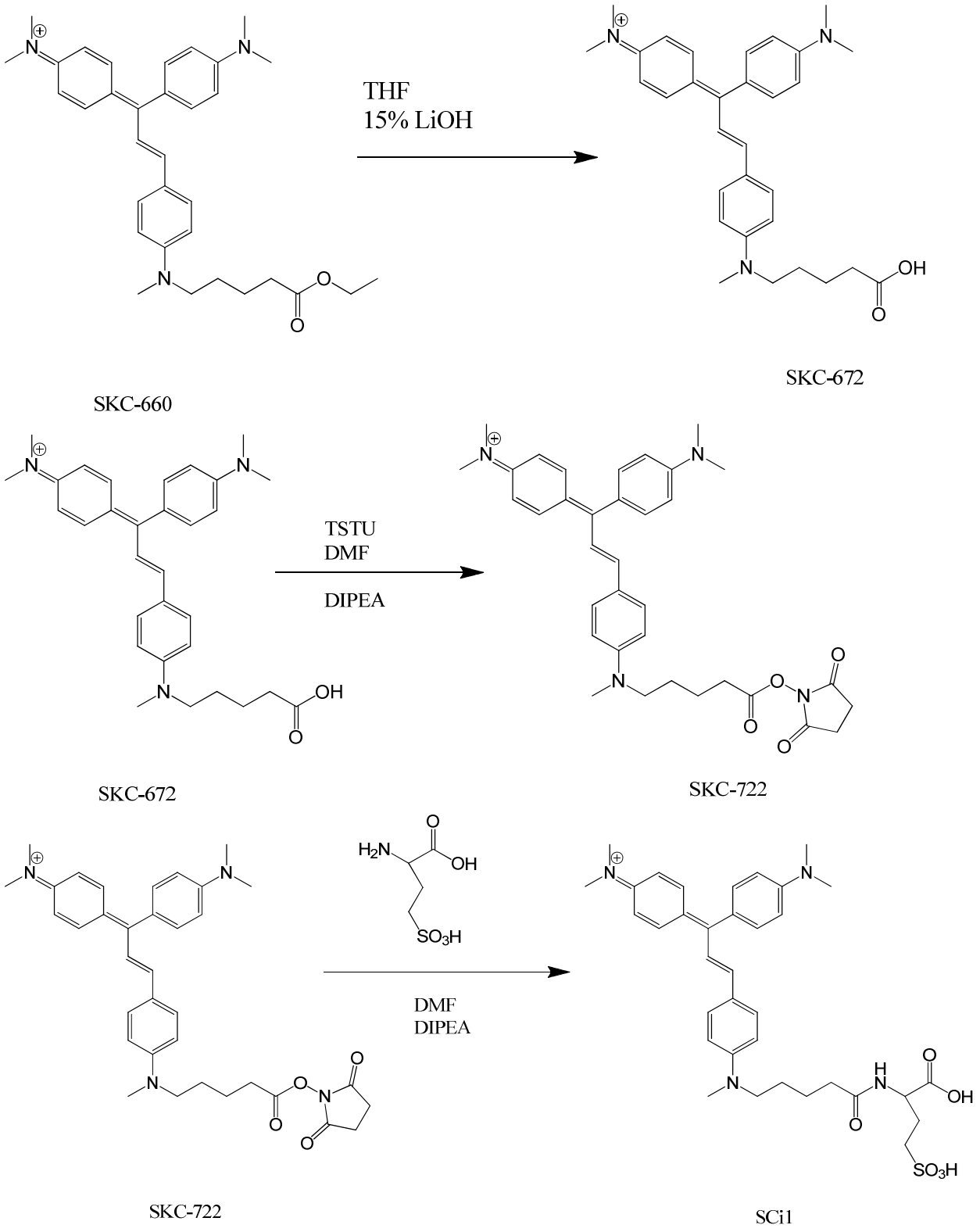
ethyl 5-((4-formylphenyl)(methyl)amino)pentanoate

SKC-659

(*E*)-*N*-(4-(1-(4-(dimethylamino)phenyl)-3-(4-((5-ethoxy-5-oxopentyl)(methyl)amino)phenyl)allylidene)cyclohexa-2,5-dien-1-ylidene)-*N*-methylmethanaminium

SKC-660

Supplemental Figure 9. Reaction scheme for synthesis of SKC-660.



Supplemental Figure 10. Synthesis scheme for SCi1 from SKC-660.