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Materials and methods

S1.1 Materials

Chemical reagents, as well as Fetal Bovine Serum (FBS) and Heat-Inactivated FBS (HI-FBS), were purchased from Sigma-Aldrich. Cell culture reagents were purchased from Life Technologies. The RAW-Blue 264.7 Macrophages, Zeocin, and QUATI-Blue reagent were purchased from Invivogen. The p65-DsRed fibroblasts were a gift from the lab of Prof. Markus Covert.

Agonists were stored in DMSO, maintained at -80 °C with a minimal number of freeze/thaw events.

S1.2 Cell culture

RAW-Blue 264.7 Macrophages were cultured in complete culture media, consisting of Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% FBS, 10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 μg/mL amphotericin B, and 200 μg/mL Zeocin. Cells were maintained at 37 °C and 5% CO_2 and used within passage 4-8 for all experiments. Complete test media for these cells was composed of DMEM (high glucose) supplemented with 10% HI-FBS.

Fibroblasts were cultured in complete culture media, consisting of DMEM (high glucose) supplemented with 20% FBS, 10,000 U/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B. Cells were maintained at 37 °C and 5% CO_2. Complete test media for these cells was composed of DMEM with 4.5 g/L glucose and 1% HI-FBS.

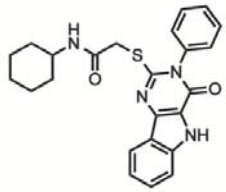
S1.3 Methods

NMR spectra were obtained on Bruker Advance 500 MHz spectrometer. UV/Vis spectra were obtained using a Thermo Fisher Scientific NanoDrop 2000c spectrophotometer. HPLC data was obtained using an Agilent 1260 Inifinity LC system. Absorbance for QUANTI-Blue measured on a Bio-Tek Quant microplate spectrophotometer MQX200 operated at 620 nm. Cells were visualized on a Zeiss 780 confocal microscope with a stage incubator adapter.

The light source for light-controlled experiments was a long-wave UV (365 nm) hand-held lamp (15 W, Blak Ray®). Masks for pinhole exposure were made using a laser cut printer (VLS2.30, Universal laser systems).

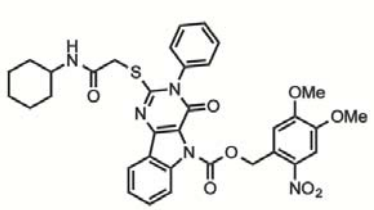
Synthesis and characterization of caged TLR4 agonist

S2.1 TLR4 agonist synthesis



The TLR4 agonist, pyrimido[5,4-*b*]indole **1** was synthesized and purified as described previously.[1] TLC (DCM/5% MeOH) Rf=0.20. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.12-1.28 (m, 5 H), 1.53-1.75 (m, 5 H), 3.53 (broad s, 1 H), 3.91 (s, 2 H), 7.28 (t, J = 7.28 Hz, 1 H), 7.48-7.63 (m, 6 H), 8.09 (d, J = 8.09 Hz, 1 H), 8.20 (d, J = 8.19 Hz, 1 H), 12.13 (s, 1 H). ^{13}C NMR (500 MHz, DMSO-d6) δ ppm 24.93, 25.66, 32.85, 37.25, 48.44, 113.35, 119.75, 120.55, 120.84, 127.80, 130.04, 130.35, 136.56, 137.72, 139.41, 153.03, 155.44, 166.22. HRMS calc'd for $C_{24}H_{24}N_4O_2SNa$ $(M + Na)^+$, 455.1512; found, 455.1511.

S2.2 Caged TLR4 agonist synthesis



In a flame-dried flask, **1** (200 mg, 0.46 mmol) was combined with NaH (60% dispersed in mineral oil, 100 mg, 2.5 mmol) in 2 mL dry THF and stirred at room temperature for 30 min. 4,5-dimethoxyl-2-nitrobenzyl chloroformate (251 mg, 0.91 mmol) was dissolved in 10 mL dry THF and added to the reaction flask. The mixture was heated to reflux for 3 h. The reaction was quenched with water and the crude product was extracted with methylene chloride. The product was purified by column chromatography with methylene chloride/20% acetonitrile to obtain 164 mg **2** (53.1% yield). TLC (DCM/5% MeOH) Rf=0.35.
^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.11-1.15 (m, 3 H), 1.31-1.37 (m, 2 H), 1.58-1.66 (m, 3 H), 1.92-1.93 (m, 2 H), 3.68 (s, 3 H), 3.79-3.81 (m, 1 H), 3.88 (s, 2 H), 3.98 (s, 1 H), 5.95 (s, 2 H), 7.01 (d, J = 7.01 Hz, 1 H), 7.38-7.40 (m, 2 H), 7.54 (t, J = 7.54 Hz, 1 H), 7.63-7.64 (m, 3 H), 7.72-7.75 (m, 2 H), 7.80 (s, 1 H), 8.13 (d, J = 8.13 Hz, 1 H), 8.44 (d, J = 8.451 H). ^{13}C NMR (500 MHz, DMSO-d6) δ ppm 24.63, 25.43, 29.76, 32.92, 36.32, 48.57, 56.38, 56.84, 67.18, 107.78, 111.41, 116.25, 117.83, 120.58, 123.00, 124.47, 126.57, 129.05, 130.00, 130.54, 130.89, 135.62, 139.53, 140.34, 144.92, 148.11, 150.96, 153.91, 153.97, 159.70, 167.10.
HRMS calc'd for $C_{34}H_{33}N_5O_8SNa$ $(M + Na)^+$, 694.1948; found, 694.1942.

S2.3 UV-Vis

The uncaging efficiency of **2** was examined by UV absorption (**Fig. 2A**) and HPLC (**Fig. 2B**) of the TLR4 agonist **1** and caged agonist **2** before and after UV exposure. Solutions for both methods were prepared in 10:1 (v:v) DMSO:H_2O. Samples were exposed to direct light (15 W, 365 nm), with the lamp positioned 1 cm from the sample.

S2.4 HPLC

To characterize the major species in solution, we performed HPLC analysis of **1**, **2** before and after UV exposure (**Fig. S1**). Samples were eluted using an acetonitrile and water gradient varying from 10:90 to 90:10 (ACN:H_2O) over 20 min at a rate of 5 mL/min. The detector was set at 250 nm for the signal acquisition, consistent with the UV-Vis characterization that showed local maxima at 250 nm for both **1** and **2** with and without UV light exposure. **1**, **2** had elusion times of 13.5 and 17.1 min, respectively. Confirmation of these LC peaks were performed using mass spectrometry. The compound with an elusion time of 13.5 min corresponded to ($M_{TLR4\ agonist}$+1) = 433, and the compound with an elusion time of 17.1 min corresponded to ($M_{caged\ agonist}$+1) = 640. The amounts of **1** and **2** was determined by integrating the area of LC trace corresponding to each species. Percent conversion (**Fig. 1C**) was calculated using these integrated values. The 30 min deprotection experiment was performed in replicates of three. HPLC and MS characterization detected side products and we wanted to confirm the extent of the reaction. To determine the yield of the reaction and the extent of side reaction, an internal standard, methyl indole-5-carboxylate, was added at 100 μM to each sample before HPLC analysis with 200 μM of **1** (**Fig. S2**). We then quantified the amount of starting material and product formed from the photo-cleavage reaction finding that ~80% is converted to the major product with ~20% as remaining starting material and converted byproducts after 30 mins.

The reaction yielded a high amount of **2** after photo-deprotection, but there were observable side products. For photo-deprotection and the potential side products, please refer to Bochet for a review of photolabile protecting groups, including the mechanism of NVOC deprotection.[2]

Visualization of NF-κB activation in p65-DsRed fibroblasts

S3.1 Microscopy

Fibroblasts expressing p65-DsRed and H2B-GFP-expressing were visualized using both GFP and DsRed fluorescence channels. Images were obtained in 15 min increments for time-course experiments (**Fig. S5**). For subsequent experiments, images were taken at the determined time of peak activation (t=45-60 min, **Fig. 4-5**). The acquisition parameters (image size, pixel dwell, laser power, and gain) were kept constant for all experiments.

S3.2 Image analysis

The degree of NF-κB activation was determined by calculating the nuclear intensity of DsRed using ImageJ analysis of fluorescent images. The raw images were split into red and green fluorescent channels (**Fig. S7 A**). The fluorescence of the H2B-GFP (green channel) was used to set the limits of the nuclei. The green fluorescent channel was first processed by noise despeckling, median filter (2 px), and Gaussian blur (2 px) (**Fig. S7 B**). The threshold of the green fluorescent channel was set (**Fig. S7 C**) and watershed (**Fig. S7 D**) to create a binary image of individual particles (nuclei). Particles in this channel were measured (**Fig. S7 E**), with the minimum particle size set equal to the minimum pixel area of a nuclei (varies between images sizes). To determine the amount of p65-DsRed in the nucleus, the measurement (mean intensity) was then redirected to the red fluorescent channel (**Fig. S7 F**). The mean intensity of the entire red fluorescence image was also measured in order to normalize data and compare time points.

The nuclei mean intensity was averaged and normalized to the mean image intensity.

Supplemental figures

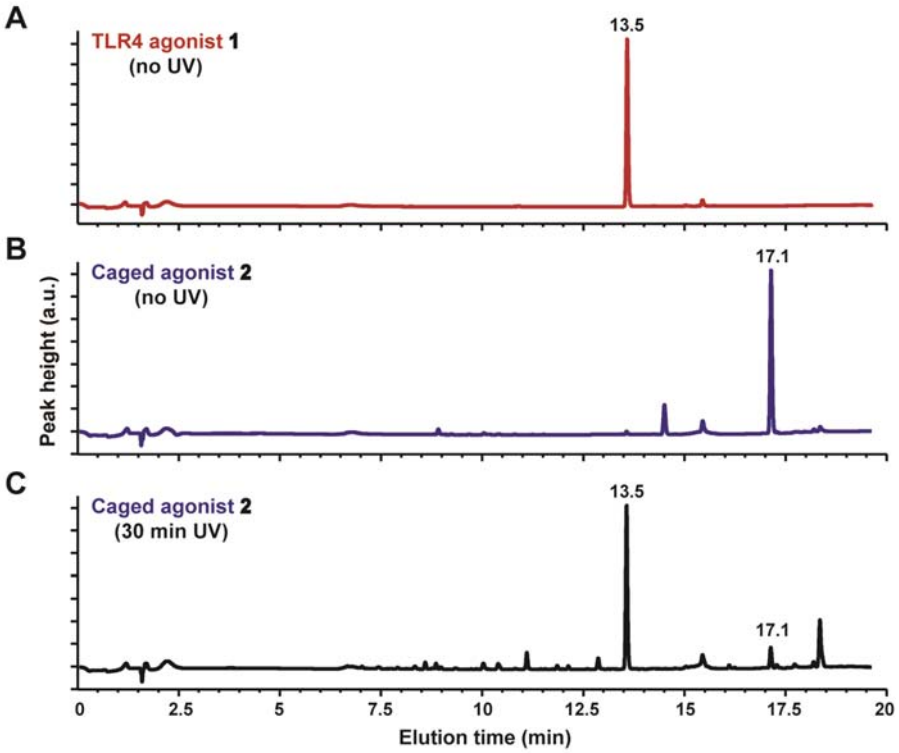


Figure S1. HPLC traces of **1** (**A**, red line) and **2** before (**B**, blue line) and after 30 min UV exposure (**C**, 15 W, 365 nm, black line). Samples were eluted using an acetonitrile and water gradient varying from 10:90 to 90:10 (ACN:H_2O) over 20 min at a rate of 5 mL/min. The detector was set at 250 nm for the signal acquisition. **1** had an elusion time of 13.5 min (**A**), and **2** had an elusion time of 17.1 min (**B**). Mass spectrometry was performed to confirm the major species in solution: **1** corresponded to ($M_{TLR4\ agonist}$+1) = 433, and **2** corresponded to ($M_{caged\ agonist}$+1) = 640. The amounts of **1** and **2** in solution exposed to UV light (**C**) was determined by integrating the area of LC trace corresponding to each species.

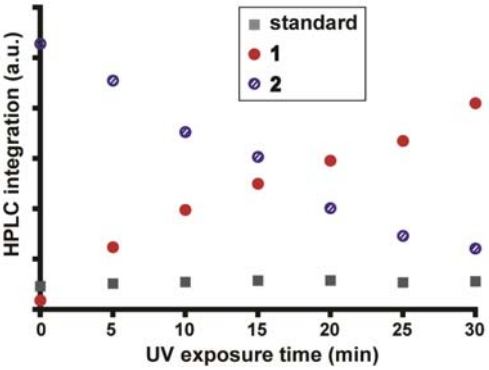


Figure S2. HPLC integrations of the major components in a solution of **2** after 30 min UV exposure (15 W, 365 nm). Aliquots were taken every 5 min of light exposure and combined with an internal standard, methyl indole-5-carboxylate, then chararcterized by HPLC analysis. Integrations corresponding to the eluted compounds (refer to Fig. S1) are plotted: **1** (red circle), **2** (blue striped circle), and the internal standard (gray square).

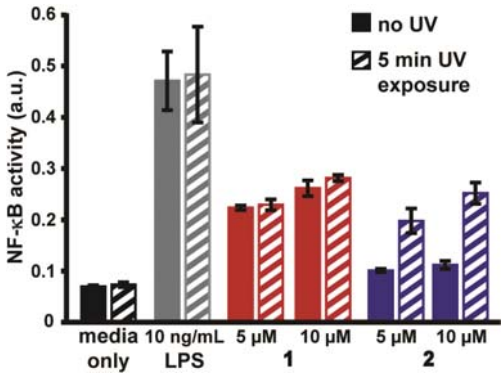


Figure S3. Light-controlled NF-κB activation in reporter cells. RAW-Blue macrophages were treated with media only (black), LPS (10 ng/mL; gray, **1** (5 and 10 μM; red), **2** (5 and 10 mM; solid blue), without (solid) or with (striped) UV light exposure (5 min, 15W, 265 nm). NF-κB activation was measured by taking the OD at 620 nm. Each experiment was performed in replicates of five.

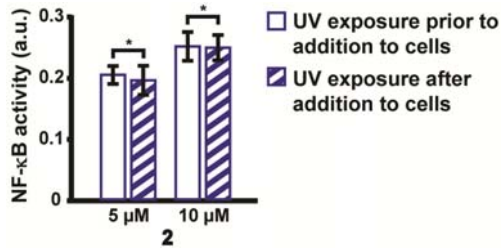


Figure S4. Effect of UV light exposure on light-controlled NF-κB activation in reporter cells. RAW-Blue macrophages were treated with **2** (5 and 10 μM) with UV light exposure prior to addition of cells (solid white), or in the presence of cells (striped). NF-κB activation was measured by taking the OD at 620 nm. Each experiment was performed in replicates of five (*$p < 0.05$).

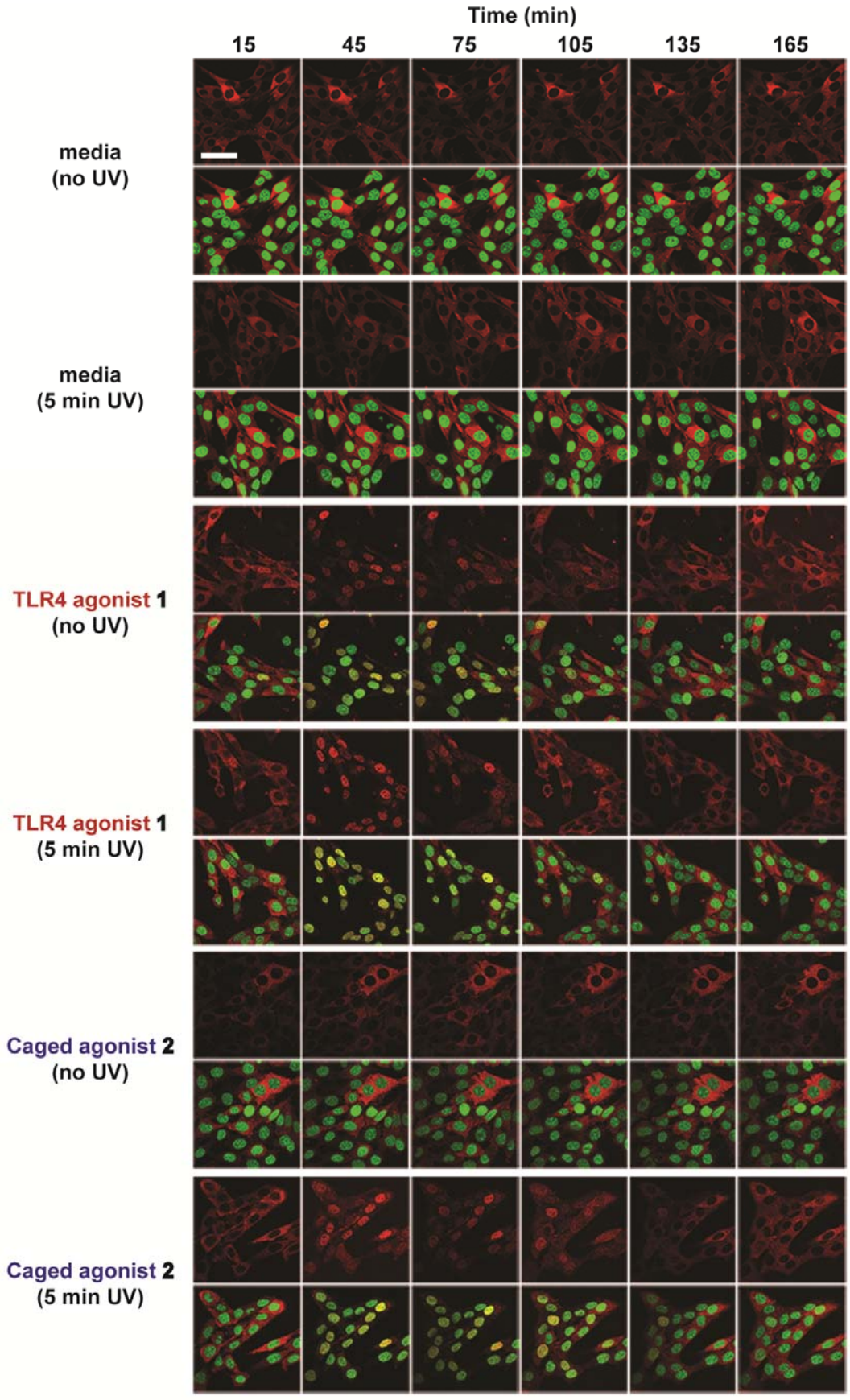

Time (min)
15
45
75
105
135
165
media
(no UV)
media
(5 min UV)
TLR4 agonist 1
(no UV)
TLR4 agonist 1
(5 min UV)
Caged agonist 2
(no UV)
Caged agonist 2
(5 min UV)

Figure S5. Time course of NF-κB activation in fluorescent reporter cells. Fibroblasts expressing p65-DsRed (NF-κB) and H2B-GFP (nuclei) were treated at t=0 with media, **1** or **2**, with and without 5 min light exposure (15 W, 365 nm), and images by fluorescence microscopy for up to 3 h. Scale bar is equal to 50 μm.

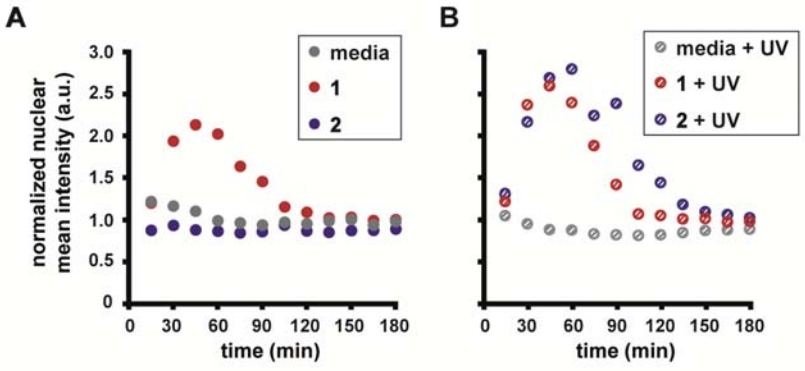


Figure S6. Quantification of NF-κB activation in fluorescent reporter cells. The red nuclear intensity was determined by ImageJ analysis for cells treated with media, **1** or **2**, without (**Fig. S6A**) and with 5 min light exposure (15 W, 365 nm, **Fig. S6B**).

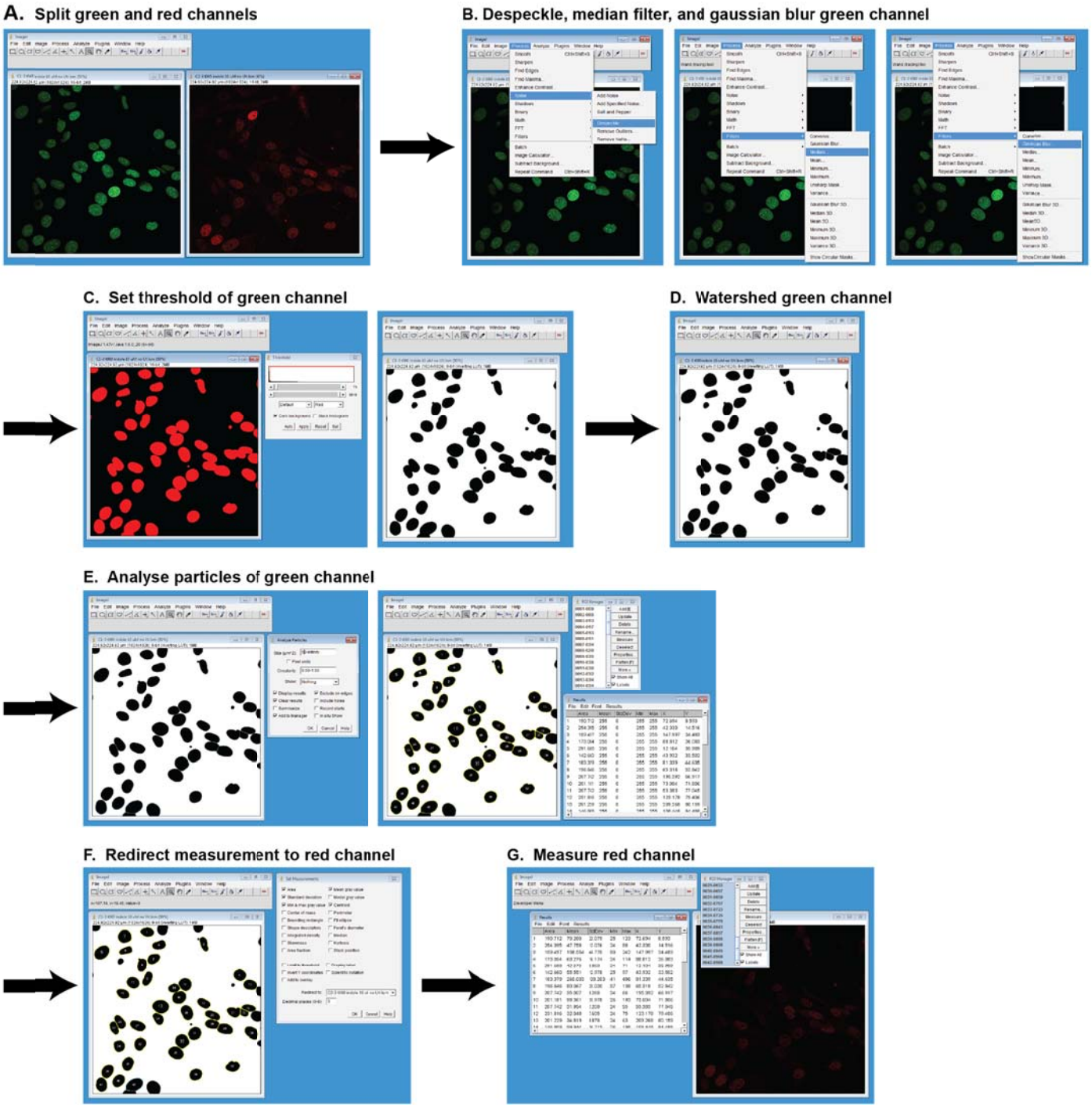


Figure S7. Flow diagram of ImageJ analysis for quantifying NF-κB activation in fluorescent reporter cells. The fluorescence of the H2B-GFP (green fluorescent channel) was used to set the limits of the nuclei (**Fig. S7 B-E**). The amount of p65-DsRed in the nucleus is determined by redirecting the measurements to the red fluorescent channel (**Fig. S7 F-G**).

References

[1] M. Chan, T. Hayashi, R. D. Mathewson, A. Nour, Y. Hayashi, S. Yao, R. I. Tawatao, B. Crain, I. F. Tsigelny, V. L. Kouznetsova, et al., *J. Med. Chem.* **2013**, *56*, 4206–4223.
[2] C. G. Bochet, *J. Chem. Soc. [Perkin 1]* **2002**, 125–142.