Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63162R2
Full Title:	Visualization of inflammatory caspases induced proximity in human monocyte-derived macrophages.
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Houston, Texas, USA
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TITLE:

Visualization of Inflammatory Caspases Induced Proximity in Human Monocyte-Derived Macrophages.

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SUMMARY:

This protocol describes the workflow to obtain monocytes-derived macrophages (MDM) from human blood samples, a simple method to efficiently introduce inflammatory caspase Bimolecular Fluorescence Complementation (BiFC) reporters into human MDM without compromising cell viability and behavior, and an imaging-based approach to measure inflammatory caspase activation in living cells.

ABSTRACT:

Inflammatory caspases include caspase-1, -4, -5, -11, and -12 and belong to the subgroup of initiator caspases. Caspase-1 is required to ensure correct regulation of inflammatory signaling and is activated by proximity-induced dimerization following recruitment to inflammasomes. Caspase-1 is abundant in the monocytic cell lineage and induces maturation of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 to active secreted molecules. The other inflammatory caspases, caspase-4 and -5 (and their murine homolog caspase-11) promote IL-1β release by inducing pyroptosis. Caspase Bimolecular Fluorescence Complementation (BiFC) is a tool used to measure inflammatory caspase-induced proximity as a readout of caspase activation. The caspase-1, -4, or -5 pro-domain, which contains the region that binds to the inflammasome, is fused to non-fluorescent fragments of the yellow fluorescent protein Venus (Venus-N [VN] or Venus-C [VC]) that associate to reform the fluorescent Venus complex when the caspases undergo induced proximity. This protocol describes how to introduce these reporters into primary human monocyte-derived macrophages (MDM) using nucleofection, treat the cells to induce inflammatory caspase activation, and measure caspase activation using fluorescence and confocal microscopy. The advantage of this approach is that it can be used to identify the components, requirements, and localization of the inflammatory caspase activation complex in living cells. However, careful controls need to be considered to avoid compromising cell viability and behavior. This technique is a powerful tool for the analysis of dynamic caspase interactions

at the inflammasome level as well as for the interrogation of the inflammatory signaling cascades in living MDM and monocytes derived from human blood samples.

INTRODUCTION:

The caspases are a family of cysteine aspartate proteases that can be grouped into initiator caspases and executioner caspases. Executioner caspases comprise caspase-3, -6 and -7. They are naturally found in cells as dimers and are cleaved by the initiator caspases to execute apoptosis[1]. Initiator caspases include human caspase-1, -2, -4, -5, -8, -9, -10 and -12. They are found as inactive zymogens (pro-caspases) that are activated by proximity-induced dimerization and stabilized by auto-proteolytic cleavage[2,3]. The inflammatory caspases are a subset of the initiator caspases[2] and encompass caspase-1, -4, -5, and -12 in humans, and caspase-1, -11, and -12 in mouse[4,5]. Rather than an apoptotic role, they play a central role in inflammation. They mediate proteolytic processing and secretion of pro-interleukin (IL)-1β and pro-IL-18[6,7], which are the first cytokines to be released in response to pathogenic invaders[8,9]. Caspase-1 is activated upon recruitment to its activation platform; a large molecular weight protein complex termed the inflammasome (**Figure 1A**)[10]. Dimerization of caspase-4, -5, and -11 occurs independently of these platforms through a noncanonical inflammasome pathway[11,12].

Canonical inflammasomes are cytosolic multimeric protein complexes that consist of an inflammasome sensor protein, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and the effector protein caspase-1[10]. The most well studied canonical inflammasomes are the NOD-like receptor family containing a pyrin domain (NLRP), NLRP1 and NLRP3, the NLR family containing a CARD (NLRC), NLRC4, and the absent in melanoma 2 (AIM2). They each contain a pyrin, a CARD, or both domains. The CARD domain mediates the interaction between CARD-containing caspases and their upstream activators. Therefore, the scaffold molecule ASC, which is composed of an N-terminal pyrin domain (PYD) and a C-terminal CARD motif[13,14], is required for recruitment of caspase-1 to the NLRP1[10], NLRP3[15], and AIM2[16] inflammasomes.

Each inflammasome is named after its unique sensor protein that recognizes distinct pro-inflammatory stimuli (**Figure 1B**). Activators of this pathway are termed canonical stimuli. Inflammasomes serve as sensors for microbial components and tissue stress and assemble to trigger a robust inflammatory response through activation of the inflammatory caspases[17]. Inflammasome assembly initiates caspase-1 activation to mediate maturation and secretion of its main substrates pro-IL-1β and pro-IL-18. This process occurs via a two-step mechanism. First, a priming stimulus upregulates the expression of certain inflammasome proteins and pro-IL-1β through activation of the NF-κB pathway. Second, an intracellular (canonical) stimulus induces inflammasome assembly and recruitment of procaspase-1[6,7].

Caspase-4 and caspase-5 are the human orthologs of murine caspase-11[11]. They are activated in an inflammasome-independent manner by intracellular lipopolysaccharide (LPS), a molecule found in the outer membrane of Gram-negative bacteria[18–20], and by extracellular heme, a product of red blood cell hemolysis[21]. It has been proposed that LPS binds directly to the CARD motif of these proteins and induces their oligomerization[20]. Activation of caspase-4 or caspase-5

promotes IL-1β release by inducing an inflammatory form of cell death called pyroptosis through cleavage of the pore-forming protein gasdermin D (GSDMD)[18,19]. In addition, the efflux of potassium ions resulting from caspase-4 and GSDMD-mediated pyroptotic death induces activation of the NLRP3 inflammasome and subsequent activation of caspase-1[22,23]. Therefore, caspase-4, -5, and -11 are considered intracellular sensors for LPS that are able to induce pyroptosis and caspase-1 activation in response to specific stimuli[11,24].

[Place **Figure 1** here]

Measuring specific initiator caspases activation is difficult, and there are not many methods available to do so by imaging approaches. Caspase Bimolecular Fluorescence Complementation (BiFC) can be used to visualize inflammatory caspase activation directly in living cells (**Figure 1A**)[25]. This technique has been recently adapted for use in human monocyte-derived macrophages (MDM)[21]. Caspase BiFC measures the first step in inflammatory caspase activation, induced proximity to facilitate dimerization. Expression of plasmids encoding the CARD-containing caspase pro-domain fused to non-fluorescent fragments of the photostable yellow fluorescent protein Venus (Venus-C [VC]) and Venus-N [VN]) are used. When the two caspase pro-domains are recruited to their activation platform or undergo induced proximity, the two halves of Venus are brought in close proximity and forced to refold and fluoresce (see **Figure 1A**,**B**). This provides a real-time readout of specific inflammatory caspase activation.

Human MDM abundantly express inflammasome genes and pattern recognition receptors that identify danger signals and pathogen products. This provides an ideal cell type for the interrogation of inflammatory caspase pathways. In addition, they can be derived from peripheral blood and even from patient samples to assess inflammatory caspase activation in a specific disease state. This protocol describes how to introduce the BiFC caspase reporters into MDM using nucleofection, an electroporation-based transfection method, how to treat the cells to induce inflammatory caspase activation, and how to visualize the active caspase complexes using microscopy approaches. Additionally, this methodology can be adapted to determine the molecular composition of these complexes, subcellular localization, kinetics, and size of these highly ordered structures[25–27].

PROTOCOL:

This protocol follows the guidelines of Baylor College of Medicine's human research ethics committee for the manipulation of human samples. Blood samples are handled following the institutional safety guidelines for human samples. Blood samples are obtained at a regional blood bank, where they are collected with citrate phosphate dextrose (CPD) solution. However, blood collected with other anticoagulants like sodium heparin, lithium heparin, or EDTA can also be used for this protocol[28,29].

1. Isolation of human monocytes and differentiation into macrophages

1.1. Obtain anticoagulated blood from de-identified healthy individuals at a regional blood

bank and isolate peripheral blood mononuclear cells (PBMCs) as indicated below.

NOTE: Perform all steps in a tissue culture laminar flow hood. Use sterile tubes only and wear gloves. Add 10% bleach to all blood-related products when disposing of. Sterile PBS (1x) or DPBS (without Ca^{2+} and Mg^{2+}) can be used interchangeably.

1.1.1. Prepare the dilution buffer: Supplement 1x sterile PBS with 2% FBS and 0.5 mM EDTA.

1.1.2. Prepare the culture medium: Supplement RPMI-1640 medium with FBS (10% (v/v)), glutamax (2 mM), and Penicillin/ Streptomycin (50 I.U./50 µg/mL)

1.1.3. Precool the running buffer (**Table of Materials**) according to the manufacturer's protocol.

1.1.4. Dilute whole blood with two volumes of dilution buffer. Using a serological pipet, transfer 15 mL of the anticoagulated blood to a 50 mL tube containing 30 mL of the dilution buffer. Mix gently by inversion.

1.1.5. For each 10 mL of whole blood or 30 mL of diluted blood, add 15 mL of the density gradient medium to a 50 mL empty tube.

1.1.6. Layer the density gradient medium from step 1.1.5 with 30 mL of diluted blood slowly and steadily using a 25 mL serological pipet. Keep the tip of the pipet against the wall of the tube and the tube at a tilted angle.

1.1.7. Carefully transfer the tubes to a swinging-bucket centrifuge. Avoid disturbing the two phases. Centrifuge the tubes at 400 x *g* at room temperature (RT) for 25 min with acceleration and deceleration set to the minimum value.

1.1.8. Carefully remove the top (clear) plasma layer using a 10 mL pipet and dispose of in a container with bleach (10%).

1.1.9. Collect the interphase (white) layer of peripheral blood mononuclear cells (PBMCs, **Figure 2**) with a 10 mL pipet and transfer to a fresh 50 mL tube. Combine in each 50 mL tube up to 30 mL of the white layer from different tubes of the same donor.

1.1.10. Bring each tube to a total volume of 50 mL with the dilution buffer from step 1.1.1 and centrifuge at 300 x *g* and 4 °C for 10 min. Remove the supernatant with a 10 mL pipet and dispose of it in a container with bleach (10%).

1.1.11. Resuspend each cell pellet in 1 mL of the pre-cooled running buffer from step 1.1.3 using a p1000 micropipette. Combine cell suspensions from the same donor in a new 15 mL tube. Bring the volume of each tube to 15 mL with pre-cooled running buffer and mix well by inversion.

1.1.12. Take a 20 µL aliquot of the cell suspension from step 1.1.11 and prepare a 1:100 dilution

using 1x sterile PBS. Determine the cell number using a hemocytometer.

1.1.13. Centrifuge the cell suspension from step 1.1.11 at 300 x *g* and 4 °C for 10 min and remove the supernatant with a 10 mL pipet. If necessary, use a p200 micropipette to remove the supernatant completely.

1.1.14. Resuspend the isolated PBMCs in 80 µL of pre-cooled MACS running buffer for each 1 x 10^7 cells, adding up to a maximum of 800 µL of the buffer.

1.1.15. Add 20 µL of anti-human CD14 MicroBeads per each 1 x 10^7 cells or up to 100 µL per blood sample (~100 mL of undiluted blood). Mix well by inversion and place on a tube rotator for 20 min with continuous mixing at 4 °C.

1.1.16. Remove the samples from the tube rotator, add 10 mL of pre-cooled running buffer to each tube, and centrifuge at 300 x *g* (acceleration = 5, deceleration = 5) and 4 °C for 10 min.

1.1.17. Remove the supernatant with a 10 mL pipet and resuspend up to 1 x 10^8 cells in 500 µL of pre-cooled running buffer (2 x 10^8/mL).

1.1.18. Perform the isolation of CD14-positive cells by magnetic cell sorting using a manual or automated system (**Table of Materials**) per the manufacturer's instructions.

1.1.19. Take a 20 µL aliquot of the cell suspension from step 1.1.18 after CD14-positive selection and prepare a 1:100 dilution using 1x sterile PBS. Determine the cell number by counting the cells on a hemocytometer.

1.1.20. Centrifuge the CD14-positive cells at 300 x *g* and RT for 10 min. Remove the supernatant using a 10 mL pipet or a vacuum system.

1.1.21. Resuspend the cell pellet from step 1.1.20 in pre-warmed culture medium from step 1.1.2 to a final cell density of 1 x 10^7 cells/mL.

1.2. Seed the isolated CD14-positive monocytes at a cell density of 5 x 10^6 cells.

1.2.1. On a 10 cm tissue culture dish, add 10 mL of culture medium from step 1.1.2 supplemented with 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF).

1.2.2. Add 0.5 mL of the cell suspension from step 1.1.21 to the culture medium dropwise and gently swirl the plate. Incubate cells in a humidified tissue culture incubator (37 °C, 5% CO_2) overnight.

1.3. The next day, aspirate the medium using a vacuum system to remove cells that did not attach overnight. Add 10 mL of fresh culture medium supplemented GM-CSF (50 ng/mL) and incubate cells in a humidified tissue culture incubator (37 °C, 5% CO_2) for 7 days to allow complete

differentiation (see **Figure 3A** for the appearance of CD14+ monocytes at various stages of differentiation in GM-CSF). Exchange the culture medium every 2–3 days and supplement with fresh GM-CSF (50 ng/mL) each time.

[Place **Figure 2** here]

2. Preparation of electroporation components

NOTE: This protocol is designed for a 10 µL-Neon tip (**Table of Materials**). For each transfection, use 1–2 x 10^5 cells. It is recommended to seed transfected cells on a 48-well plate or 8-well chambered dish (10 µL-transfected cells per well). 1x sterile DPBS (without Ca^{2+} and Mg^{2+}) can also be used.

2.1. On day 7, prepare antibiotic-free medium by supplementing RPMI-1640 medium with FBS (10 % (v/v)) and glutamax (2 mM).

2.2. Place serum-free RPMI-1640 medium, trypsin-EDTA (0.25%) solution, 1x sterile PBS (without Ca^{2+} and Mg^{2+}), and complete culture medium from step 1.1.2 in a 37 °C water bath.

2.3. If using glass-bottomed dishes (for confocal microscopy) coat the dishes with poly-D-lysine hydrobromide.

2.3.1. Coat an 8 well-chambered dish with 200 µL of poly-D-lysine hydrobromide (0.1 mg/mL in 1x sterile PBS) and incubate for 5 min at RT.

2.3.2. Aspirate the poly-D-lysine solution and wash the glass once with 1x sterile PBS. Aspirate the PBS and proceed with step 2.4.

2.4. Add 200 µL of antibiotic-free medium per well of the 48 well plate or 8 well-chambered dish and pre-incubate in a humidified tissue culture incubator (37 °C, 5% CO_2) until ready to plate the transfected cells.

3. Preparation of cells for electroporation

NOTE: The yield of MDM from a 10 cm dish at the end of the 7-day differentiation period is approximately 1.5 x 10^6 cells. 1x sterile DPBS (without Ca^{2+} and Mg^{2+}) can also be used. This protocol was optimized so that most macrophages are detached from the plate with the maintenance of cell viability and integrity. MDM are difficult to detach from cell culture plates. Therefore, it may be necessary to perform steps 3.2 and 3.3 twice to dissociate the cells. Ensure that each incubation time with trypsin-EDTA (0.25%) does not exceed 5 min.

3.1. Aspirate the media from fully differentiated macrophages on 10 cm dishes and wash the cell monolayer with warm serum-free RPMI-1640 medium. Make sure to completely remove the medium.

3.2. Harvest the cells by adding 2 mL of warm trypsin-EDTA (0.25%) solution per 10 cm dish and incubate in a humidified tissue culture incubator (37 °C, 5% CO_2) for 5 min.

3.3. Complete the cell detachment by gently pipetting the trypsin-EDTA (0.25%) solution up and down over the entire dish area using a p1000 micropipette. Transfer the cell suspension to a 15 mL conical tube containing 5 mL of warm complete culture medium from step 1.1.2.

3.4. Take the dish to a bright field microscope and check for cell detachment in various fields of view. If there is a considerable amount of cells still attached, repeat steps 3.2–3.3.

3.5. Centrifuge the cell suspension at 250 x *g* for 5 min at RT.

3.6. Aspirate the medium and resuspend the cells in 10 mL of 1x sterile PBS pre-warmed to 37 °C. Take a 20 µL aliquot to determine the cell number using a hemocytometer.

3.7. Take 1–2 x 10^5 cells per intended transfection and place in a 15 mL tube. Bring to a final volume of 15 mL with pre-warmed 1x sterile PBS. Centrifuge at 250 x *g* for 5 min at RT.

3.8. Aspirate the PBS and centrifuge one more time for 1 min at 250 x *g*. Remove any residual PBS from the cell pellet using a p200 micropipette.

4. Nucleofection of caspase BiFC components into human monocyte-derived macrophages

NOTE: This section of the protocol is performed using the Neon Transfection System (**Table of Materials**). This protocol outlines the steps to transfect 1–2 x 10^5 cells using a 10 µL Neon tip (**Table of Materials**). If using a 100 µL Neon tip, scale up accordingly. Avoid exposing cells to resuspension buffer R for more than 15 min, as this may decrease cell viability and transfection efficiency.

4.1. Dilute the reporter plasmid (i.e., mCherry or dsRedmito at 100 ng/µL) in nuclease-free water or 0.5x TE buffer to visualize the transfected cells.

4.2. Dilute the caspase BiFC plasmids to an appropriate concentration in nuclease-free water or 0.5x TE buffer, so that the total volume of plasmids does not exceed 30% of the total transfection volume of 10 µL (i.e., 300 ng/µL of C1 Pro-VC and 300 ng/µL of C1 Pro-VN).

4.3. Prepare a 1.5 mL sterile microtube per intended transfection and add the appropriate amount of reporter plasmid (i.e., 50 ng or 0.5 µL) and caspase BiFC plasmids (i.e., 300 ng or 1 µL of C1 Pro-VC and 300 ng or 1 µL of C1 Pro-VN fragment). Keep the microtubes in the hood at all times.

4.4. Place the pipette station, device, tips, electroporation tubes, and pipette in a sterile laminar flow hood.

NOTE: The pipette station, device, tips, electroporation tubes, and pipette are included in the Neon Transfection System.

4.5. Connect the high voltage and sensor connector on the pipette station to the rear ports on the device as per the manufacturer's instructions. Keep the pipette station close to the device.

4.6. Connect the power cord to the rear AC inlet and proceed to connect the device to the electrical outlet. Press the power switch to turn on the device.

4.7. Enter the transfection parameters in the startup screen displayed when the device is switched on and appropriately connected. Press on **Voltage**, enter 1000, and press on **Done** to set the voltage to 1000 V. Press on **Width**, enter 40, and press on **Done** to set the pulse duration to 40 ms. Lastly, press on **# Pulses**, enter 2, and press on **Done** to set the number of electrical pulses to 2.

4.8. Take one of the electroporation tubes (provided in the kit) and fill it with 3 mL of electrolytic buffer E (for 10 µL tips and provided in the kit) at RT. Insert the electroporation tube into the pipette holder on the pipette station. Ensure that the electrode on the side of the tube is facing inwards and that a click sound is heard when the tube is inserted.

4.9. Take the cell pellet from step 3.8 and add 10 µL of pre-warmed resuspension R buffer (provided in the kit) for each 1–2 x 10^5 cells. Mix gently with a p20 micropipette. Add 10 µL of the cell suspension to each tube set up in step 4.3 and mix gently with a p20 micropipette.

4.10. Take the pipette and insert a tip by pressing the **Push** button to the second stop. Make sure the clamp completely picks up the mounting stem of the piston in the tip and that no gap is observed in the top-head of the pipette.

4.11. To aspirate the sample, press the **Push** button on the pipette to the first stop and dip into the first tube containing cell/plasmid DNA mixture. Slowly aspirate the mixture into the pipette tip.

NOTE: Avoid air bubbles as they can cause arcing during electroporation, and if detected by the device, can prevent delivery of the electric pulse. If air bubbles are observed, release the contents into the tube and try aspirating again.

4.12. Insert the pipette with the sample very carefully into the pipette holder. Make sure the pipette clicks and that it is properly placed.

4.13. Press **Start** on the touchscreen and wait until the electric pulses are delivered. A message on the screen will indicate completion.

4.14. Slowly remove the pipette from the station and immediately add the transfected cell

suspension into the corresponding well with pre-warmed antibiotic-free medium from step 2.4 by slowly pressing the push-button to the first stop.

NOTE: This tip can be reused up to three times for the same plasmid; otherwise, discard it into a biohazard waste container by pressing the **Push** button to the second stop.

4.15. Repeat steps 4.10–4.14 for each tube containing a cell/plasmid DNA mixture.

4.16. Gently rock the plate with transfected cells and incubate for 1–3 h in a humidified tissue culture incubator (37 °C, 5% CO_2).

4.17. Add to each well 200 µL of pre-warmed culture medium (complete medium) from step 1.1.2. Place the dish in the humidified tissue culture incubator (37 °C, 5% CO_2) again. Allow at least 24 h for gene expression.

4.18. The next day, inspect the cell viability and transfection efficiency using an epifluorescence microscope.

4.18.1. Turn on the epifluorescence microscope and the fluorescent light source box per the manufacturer's instructions and place the culture dish on the microscope stage.

4.18.2. Select the 10x or 20x objective and the 568 nm (RFP) filter.

4.18.3. To estimate cell viability, press the **Transmitted Light LED (TL)** button to visualize all cells in the selected field. While looking into the microscope eyepiece, turn the focus knob until cells are observed and check for cell attachment in the selected field.

NOTE: Fully attached cells represent the viable cells while floating cells represent non-viable cells. If the confluency of the well is high, the presence of non-attached cells could be the result of an overestimation of cell number and not the result of low viability. However, low confluency accompanied by a high content of floating cells signifies low viability that may result from arcing during electroporation, plasmid toxicity, or overexposure to resuspension R buffer. Do not use wells that display the latter behavior.

4.18.4. To estimate transfection efficiency, focus on cells in the selected field under transmitted light as described above. Count the total number of cells in the selected field. With the **Transmitted Light LED (TL)** switched off, press the **Reflected Light LED button (RL)** to switch on.

4.18.5. Fine focus on the reporter gene fluorescence (red cells) and count the total number of red fluorescent cells. Repeat these steps (4.18.4–4.18.5) for at least two more fields per well.

5. Treatment of transfected MDM and caspase BiFC data acquisition

NOTE: If planning to image the cells using an epifluorescence or confocal microscope, treatment

with qVD-OPh (20 µM) for 1 h prior treatment with the chosen stimulus is advised to prevent caspase-dependent cell death (predominantly apoptosis). This is used in imaging to prevent cells from lifting off due to apoptosis, making them very difficult to image as they move out of the focal plane. Note that caspase recruitment to the activation platform and the associated caspase BiFC is not dependent on the catalytic activity of the caspase, and consequently, caspase inhibition will not affect this step.

5.1. Treat with chosen stimulus approximately 24 h after transfection and incubate for as long as necessary for each drug.

5.1.1. Prepare imaging medium by supplementing culture medium from step 1.1.2 with Hepes (20 mM, pH 7.2–7.5) and 2-mercaptoethanol (55 µM).

5.1.2. Add the desired concentration of stimulus to the pre-warmed imaging medium and mix gently.

5.1.3. Remove the media from the cells carefully with a p1000 micropipette and add 500 µL of the stimulus solution from step 5.1.2 down the side of the well.

5.1.4. To run untreated control wells, add an imaging medium without the stimulus.

5.1.5. Incubate the cells in a humidified tissue culture incubator (37 °C, 5% CO_2) for as long as indicated for each treatment.

5.2. Visualize the cells using an epifluorescence or confocal microscope.

5.2.1. Turn on the microscope and the fluorescent light source, following the manufacturer's instructions.

5.2.2. Select the 10x or 20x objective and place the culture dish on the microscope stage.

5.2.3. Using the microscope eyepiece, find cells under the 568 nm filter and focus on the cells expressing the dsRedmito/mCherry reporter (red cells).

5.2.4. Count all the red cells in the visual field and record the number.

5.2.5. While in the same visual field, change to the 488 or 512 filter (GFP or YFP), proceed to count the number of red cells that are also green (Venus-positive or BiFC-positive) and record the number.

5.2.6. Count at least 100 dsRedmito/mCherry -positive cells from a minimum of three individual visual fields.

5.2.7. Calculate the percentage of Venus-positive transfected cells per visual field and average

the resulting percentages for each treatment (well) to get the standard deviation.

5.3. Image the cells using an epifluorescence or confocal microscope

NOTE: To acquire confocal images using a 20x objective or a greater magnification, cells should be plated on glass dishes unless the microscope is equipped with a long pass objective.

5.3.1. Follow steps 5.2.1–5.2.3. If using a confocal microscope with the 40x, 60x or 63x oil objective, place a drop of oil on the objective.

5.3.2. Visualize the live image of the cells on the computer screen as acquired by the camera. Use the epifluorescence light source for fluorescent images or switch the light source to the lasers for confocal images.

5.3.3. Fine-tune the focus and position of the cells using the joystick control and focus wheel.

5.3.4. Set the percentage laser power and exposure time for the 512 nm or 488 nm (YFP or GFP) and 568 nm (RFP) lasers so that the signal in the image looks good and does not reach saturation.

5.3.5. Turn on the live capture and examine the resulting image. Ensure that a distinct peak is seen for each fluor in the display histograms for both channels.

5.3.6. Adjust the laser power and exposure time as necessary. Keep these values as low as possible while still being able to detect both fluorescent signals (RFP and GFP/YFP).

5.3.7. While visualizing the live image of the cells, take multiple representative images of a field that contains one or more cells expressing the mCherry/dsRedmito reporter for each well of the plate and save the data.

REPRESENTATIVE RESULTS:

The scheme shown in **Figure 2** gives an overview of how to obtain, transfect, and image human MDM. After incubation of the selected CD14+ monocytes with GM-CSF for 7 days, the cell morphology changes over the course of the differentiation period (**Figure 3A**), going from spherical suspension cells to spindly and fully attached (days 3 and 4), and lastly to more spread cells when fully differentiated (day 7). Fully differentiated cells are then detached from the plate and transfected with the caspase BiFC pairs (VC and VN) along with the reporter plasmid (e.g., dsRedmito, a plasmid encoding a red fluorescent protein targeted to the mitochondria), which is used to label the transfected cells (**Figure 3B**) and employed to assess the efficiency of the transfection after 24 h. **Figures 3B–D** show an example of BiFC results using the caspase-1 pro BiFC transfected cells treated for 20 h with nigericin (5 µM), a known pro-inflammatory stimulus that triggers the assembly of the NLRP3 inflammasome[30,31]. Untreated cells show no Venus fluorescence (**Figure 3B**), and in nigericin-treated cells, caspase-1 BiFC appears as a single punctum with the typical shape of ASC specks[32,33,25] (**Figure 3C**). **Figure 3D** shows an example of quantitation of Venus-positive MDM. The highest percentage of caspase-1 BiFC is seen in the LPS

+ nigericin treatment group (**Figure 3D**). This result is consistent with activation of a canonical inflammasome, where both a priming signal (LPS) and an intracellular signal (nigericin) are required for activation of capase-1.

[Place **Figure 3** here]

An example of plasmid titration is shown in **Figure 4**, where increasing amounts of each BiFC plasmid is transfected. This allows for the selection of the optimal dose of plasmid, resulting in a specific signal and minimal background. **Figure 4A** shows the results of human MDM transfected with increasing concentrations of the caspase-1, -4, and -5 pro BiFC pairs (VC and VN) treated with heme, a highly pro-inflammatory molecule that results from red blood cell destruction[34]. The highest percentage of Venus-positive cells for caspase-1, -4, and -5 pro BiFC pairs results from 400 ng, 500 ng, and 1000 ng of transfected plasmid, respectively. However, using the highest amount of plasmid also runs the risk of increasing non-specific background (**Figure 4B**). For example, transfection of 1000 ng of the caspase-5 pro BiFC pair results in almost 40% non-specific background. Therefore, using the lower 700 ng amount is considered optimal for this plasmid pair (**Figure 4B**). In **Figure 4C**, representative confocal images obtained with a 20x objective of a field of cells 24 h post-transfection are shown. In this image, one can see that the untreated transfected cells are viable based on the appearance of the mitochondria (mitochondria in dead cells are highly fragmented) and the morphology of the cells (apoptotic cells are shrunken). After treatment with heme, the caspase-1 complex appears as a single green punctum similar to the one induced by nigericin in **Figure 3C,** and its appearance was accompanied by cell shrinkage.

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Inflammatory caspases and caspase-bimolecular fluorescence complementation (BiFC) assay. (**A**) Diagram showing the caspase-BiFC system, where two caspase-1 pro-domains (C1-pro) linked to each non-fluorescent fragment of Venus (Venus-C or Venus-N) are recruited to the NLRP3 activation platform, forcing Venus to refold and fluoresce. This complex appears as a green spot under the microscope and serves as a readout for inflammatory caspase-induced proximity, which is the first step in initiator caspase activation. (**B**) Schematic showing the domain organization of inflammasome components and inflammatory caspases.

Figure 2: Schematic overview of the experimental workflow.

Figure 3: Differentiation of CD14-monocytes and transfection of human MDM. (**A**) Representative bright-field images of CD14+ monocytes from peripheral blood exposed to GM-CSF at days 1, 3, 4, and 7 of differentiation (Scale bar, 100 µm). (**B–C**) Human MDM were transfected with caspase-1 pro BiFC pairs and the transfection reporter dsRedmito (50 ng, red). 24 h later, cells were primed with and without LPS (100 ng/mL) for 3 h and treated with nigericin (5 µM) in imaging medium for 20 h. Representative confocal images of untreated and nigericin-treated cells are shown (Scale bar, 10 µm). The BiFC is shown in green. **(D)** Cells from (**C**) were assessed for the percentage of dsRedmito-positive cells (red, transfected cells) that were Venus-

positive (green, caspase-1 BiFC complex) at 20 h. Error bars represent SD of at least two independent counts per well.

Figure 4: Human MDM transfected with different amounts of inflammatory caspase pro BiFC pairs. (**A**) Titration of caspase-1 pro; caspase-4 pro; or caspase-5 pro BiFC pairs. Human MDM were transfected with the indicated amounts of the caspase pro BiFC pairs with dsRedmito as a transfection reporter (50 ng) and incubated overnight. On the next day, the culture medium was removed from all wells, and cells were treated with and without heme (50 µM) in 0.1% FBS medium. After 1 h, an equal amount of complete medium was added to all wells to reconstitute the FBS concentration to 5% and prevent heme from killing the cells. After a total treatment of 20 h, cell were assessed for the percentage of dsRedmito-positive transfected cells that were Venus-positive, determined from a minimum of 300 cells per well. Error bars represent SD of at least three independent counts per well. (**B**) Human MDM were transfected with selected amounts of caspase-1 pro; caspase-4 pro; or caspase-5 pro BiFC pairs, along with dsRedmito (50 ng) as a reporter for transfection. 24 h post-transfection, cells were treated with or without heme (50 µM) in 0.1% FBS. After 1 h, FBS was reconstituted to 5% to inhibit extracellular heme. Cells were assessed for the percentage of dsRedmito-positive cells (red, transfected cells) that were Venus-positive (green, caspase BiFC complex) at 20 h determined from a minimum of 300 cells per well. Results are represented as percentage Venus-positive cells. Error bars represent SD of at least three independent counts per well. (**C**) Representative confocal images obtained with a 20x objective of a field of cells 24 h post-transfection and treated with and without heme as described in (**B**). Red: dsRedmito-positive cells; Green: Venus-positive cells; Scale bar, 20 µm.

DISCUSSION:

This protocol describes the workflow to obtain macrophages from monocytes isolated from human blood samples and a method to efficiently introduce the inflammatory caspase BiFC reporters into human MDM without compromising cell viability and behavior.

This protocol takes advantage of the BiFC technique[35] to tag the inflammatory caspases at the caspase recruitment domain (CARD) with non-fluorescent fragments of the split fluorescent protein Venus. Inflammatory caspases encode a catalytic domain composed of a large (p20) and a small (p10) subunit, and a conserved protein-protein interaction motif termed a CARD at their N-terminus or pro-domain[36] (**Figure 1B**). Recruitment of inflammatory caspases to their activation platforms is dependent on an intact CARD. For this approach, two caspase pro-domain constructs (encoding the CARD motif) are generated, each linked to the non-fluorescent fragments of the photostable yellow protein Venus (Venus-C [VC]) and Venus-N [VN]) along with a fluorescent transfection reporter to allow visualization of the cells prior to or in the absence of activation of the caspase BiFC reporter. Fluorescence is observed only when interacting proteins come into close enough proximity that would allow for dimerization and activation (see **Figure 1A**). The efficiency of this process can be quantified using a standard epifluorescence or a confocal microscope. In combination with single-cell imaging, this protocol allows visualization of asynchronous events that may be undistinguishable in bulk populations, and depending on the resolution of the images, can be used to determine the size and localization of the BiFC complex associated with each caspase.

This protocol can be adapted to a range of proteins that are activated by oligomerization, and its applicability is not limited to microscopy methodologies. This approach also allows for the investigation of differential requirements for inflammatory caspases assembly. For example, siRNA can be used to identify upstream components/requirements for specific inflammatory caspase activation. The caspase-BiFC probes and the siRNA oligo (used to silence a target protein) can be transfected simultaneously into MDM. This can identify potential protein partners or essential components of each activation platform. For example, a siRNA oligo that targets the ASC adaptor molecule, which is required for assembly of the NLRP1, NLRP3, and AIM2 inflammasomes, was used to show that heme-induced activation of caspase-1 required these canonical inflammasomes while caspase-4 and -5 did not[21]. This methodology can also be adapted to time-lapse microscopy, so the kinetics of the inflammatory caspase activation can be determined in single cells. Using such an approach, one can assess when caspase dimerization occurs by precisely detecting the timing of appearance of the BiFC onset relative to the time when the cell undergoes cell death by monitoring loss of fluorescence of the transfection reporter (mCherry) as an indication of cell lysis. There are multiple ways to acquire and analyze caspase BiFC data; the choice will depend on the question to be answered and the kind of microscope and software available. For instance, single-time point data used to determine the efficiency of activation as well as time-lapse analysis can be especially effective if an auto-acquisition feature provided by some microscope software is available. This allows objective imaging, as predetermined positions arrays are acquired from each well of a multi-well plate. Images can be quantitated both by visual inspection and by using automated imaging software like CellProfiler, ImageJ, or MATLAB to increase accuracy and objectivity.

Investigating caspase pathways in primary cells is essential to fully understand the physiological roles of these proteins. In addition, because caspase-4 and caspase-5 are replaced by a single caspase-11 in mice, being able to assess human cells is essential. However, due to low transfection efficiencies and toxicities associated with many delivery systems in primary cells, it can be difficult to maximize the use of available plasmid-based reporters. The inflammatory caspase BiFC protocol describes a simple method to transfect MDM from peripheral blood of either patients or healthy subjects, reducing the number of cells required per transfection. This allows researchers to perform more complex experiments with precious cells like macrophages without compromising cell viability and cellular properties. The ability to use patient samples provides a major advantage of being able to assess inflammatory caspase activation in specific disease states. Indeed, with some minor optimizations, this protocol could be easily adapted to other murine or human primary cells.

This protocol uses an electroporation-based approach to transfect MDM. The Neon system is particularly advantageous since it does not induce an IFN-γ response in MDM, which could disrupt the specific immune response of the cells[37]. This is a small-scale electroporation system that uses a 10 µL transfection volume with a high cell density to enable the efficient transfer of exogenous DNA to a large number of cells. It creates temporary pores in the cell membrane so that plasmids can pass quickly, avoiding the late endocytosis that occurs during chemical transfection and that can trigger plasmid degradation. This protocol uses an organic acid-based

buffer formulation (buffer R) that introduces minimal cell toxicity when compared to other buffers with chloride ions[38]. The small volume and the design of the pipette tip chamber aid in generating a uniform electric field to maintain physiological conditions resulting in high survival rates. Transfection efficiency and cell survival are highly dependent on the chemical composition of the buffer, the cell density, the strength of the pulse, and the plasmid concentration[39]. Therefore, it is essential to optimize these conditions for each cell type and plasmid introduced. In MDM, lower voltages with longer pulse durations delivered plasmids more efficiently while keeping cells viable and healthy. Consequently, the settings used for this protocol were 1000 V and 2 pulses of 40 ms that resulted in transfection efficiencies between 40–60% with cell viability higher than 90% based on the ability of the cells to re-attach to the plate after transfection. While this protocol is optimized for human MDM, varying the voltage settings from 500 V to 1700 V, increasing the number of pulses from 1 to 3, and testing length of pulses ranging from 10 to 40 ms can balance transfection efficiency and cell viability in additional cell types and experimental settings.

There are several important considerations for optimal employment of the BiFC technique. 1) It is important that the user determines the optimal amount of each caspase plasmid to be introduced, as this may vary for different proteins and cell types. This is required to ensure maximal sensitivity and specificity of the assay. It is advised to run a pilot titration of the caspase BiFC plasmids (VC and VN fragments) between 200 ng and 1000 ng as shown in **Figures 4A**–**B**. The optimal range of plasmid introduced differs for each individual caspase, and therefore, they must be individually assessed. Choose a plasmid amount that provides the highest specific signal with minimal background. Ideally, the background level should be less than 10%, but up to 20% can be used if the specific signal is high. It is also critical to avoid harsh conditions and excessive manipulation of these cells during differentiation, transfection, and treatment. This can also result in spontaneous activation, and high background levels of caspase activation that can obscure the specific results since activation of immune cells such as monocyte or macrophages is a natural process, where they can acquire pro-inflammatory functions[40]. If using patient cells, it can be difficult to control for some inherent variation in levels of inflammasome activation and genetic background that can sensitize or desensitize them to stimuli and changes in their local microenvironment. For example, macrophage function can be affected in immune-compromised individuals or individuals fighting an infection, as this type of cells play important homeostatic functions and are involved in the control of inflammation, among other functions. These genetic and environmental factors need to be considered when determining if a high background is non-specific or a biological effect. Using closely matched healthy controls (e.g., matched for gender and age) can help to assess the biological significance of a high background level of caspase BiFC. 2) It is critical that the VC and VN fragments are introduced at equal amounts since unequal expression of one Venus fragment could result in recruitment to the platform at higher frequencies and mask the full fluorescent signal, producing a false negative result. Therefore, the integrity and concentration of each individual plasmid should be validated before every transfection to avoid introducing different amounts of the VC and VN fragments. 3) Since this approach is based on the exogenous expression of the caspase reporters, additional controls for specificity should be included. Caspase-BiFC constructs containing single point mutations that disrupt binding between the caspase and its activation platform or silencing of one of the

components of the activation platform using siRNA can be used to confirm that the fluorescent signal is due to specific binding of the caspase to the inflammasome. This control experiment should result in decreased levels of Venus-positive cells. 4) Expression of the caspase BiFC constructs could trigger or affect downstream events such as cell death. To circumvent this, non-enzymatic versions of the caspase are recommended for the BiFC reporters, such as the caspase pro-domain or the full-length caspase where the catalytic cysteine residue is mutated and inactivated. To further control for this, control non-transfected treated and untreated wells should be included in the experiment and should show similar behavior to that of the transfected wells. 5) Endogenous caspase activity could possibly impact the BiFC readout. To control for this, one can express the caspase reporter in cells deficient for the caspase under study with the expectation that the BiFC efficiency would be the same.

One of the main drawbacks of this approach is that it does not measure caspase activation *per se,* but the induced proximity step required for activation to occur. This is a subtle but important distinction. By design, the pro-domain does not contain the catalytic domains of the caspase that actually dimerize. Therefore, the refolding and fluorescence of the Venus fragments act as a proxy for caspase dimerization as they can only refold if the caspases are close enough to dimerize. Thus, the BiFC is specifically measuring induced proximity. It is possible that posttranslational modifications in the catalytic domains could impede dimerization without affecting the BiFC signal. For example, caspase-2 is phosphorylated on its catalytic domain, and this impedes dimerization without preventing the recruitment of caspase-2 to its activation platform[41]. Hence, observations using the BiFC approach must be complemented with functional studies to confirm that the fluorescence observed is representative of caspase activation. Despite this drawback, if the controls discussed here are included, the measurement of induced proximity by caspase BiFC can provide a new accurate representation of specific caspase activation.

In conclusion, caspase BiFC is a powerful tool for the analysis of dynamic caspase interactions at the inflammasome level. This protocol allows for the interrogation of the inflammatory caspase signaling cascades in living immune cells. This approach not only provides a technique to specifically and accurately measure the first step in caspase activation but, by being applied to primary immune cells, has the potential to reveal properties of inflammatory caspases that could not be identified using conventional methods.

ACKNOWLEDGMENTS:

We thank the members of LBH's lab past and present who contributed to the development of this technique. This lab is supported by NIH/NIDDK T32DK060445 (BEB), NIH/NIDDK F32DK121479 (BEB), NIH/NIGMS R01GM121389 (LBH). Figure 2 was drawn using Biorender software.

DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1 Boatright, K. M. et al. A unified model for apical caspase activation. *Molecular Cell.* **11** (2),

529–541 (2003).
2 Pop, C., Salvesen, G. S. Human caspases: activation, specificity, and regulation. *Journal of Biological Chemistry.* **284** (33), 21777–21781 (2009).
3 Boice, A., Bouchier-Hayes, L. Targeting apoptotic caspases in cancer. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* **1867** (6), 118688 (2020).
4 Bolívar, B. E., Vogel, T. P., Bouchier-Hayes, L. Inflammatory caspase regulation: maintaining balance between inflammation and cell death in health and disease. *The FEBS Journal.* **286** (14), 2628–2644 (2019).
5 Martinon, F., Tschopp, J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell.* **117** (5), 561–574 (2004).
6 Lamkanfi, M., Dixit, Vishva, M. Mechanisms and functions of inflammasomes. *Cell.* **157** (5), 1013–1022 (2014).
7 Viganò, E. et al. Human caspase-4 and caspase-5 regulate the one-step noncanonical inflammasome activation in monocytes. *Nature Communications.* **6**, 8761 (2015).
8 Cerretti, D. P. et al. Molecular cloning of the interleukin-1 beta converting enzyme. *Science.* **256** (5053), 97 (1992).
9 van de Veerdonk, F. L., Netea, M. G., Dinarello, C. A., Joosten, L. A. Inflammasome activation and IL-1β and IL-18 processing during infection. *Trends in Immunology.* **32** (3), 110–116 (2011).
10 Martinon, F., Burns, K., Tschopp, J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular Cell.* **10** (2), 417–426 (2002).
11 Kayagaki, N. et al. Noncanonical inflammasome activation targets caspase-11. *Nature.* **479** (7371), 117–121 (2011).
12 Kayagaki, N. et al. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science.* **341** (6151), 1246–1249 (2013).
13 Masumoto, J. et al. ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *The Journal of Biological Chemistry.* **274** (48), 33835–33838 (1999).
14 Bertin, J., DiStefano, P. S. The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. *Cell Death and Differentiation.* **7** (12), 1273–1274 (2000).
15 Agostini, L. et al. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity.* **20** (3), 319–325 (2004).
16 Bürckstümmer, T. et al. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nature Immunology.* **10** (3), 266–272 (2009).
17 Latz, E., Xiao, T. S., Stutz, A. Activation and regulation of the inflammasomes. *Nature Reviews Immunology.* **13** (6), 397–411 (2013).
18 Kayagaki, N. et al. Caspase-11 cleaves gasdermin D for noncanonical inflammasome signalling. *Nature.* **526** (7575), 666–671 (2015).
19 Shi, J. et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature.* **526** (7575), 660–665 (2015).
20 Shi, J. et al. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature.* **514** (7521), 187–192 (2014).
21 Bolívar, B. E. et al. Noncanonical Roles of Caspase-4 and Caspase-5 in Heme-Driven IL-1β

749 Release and Cell Death. *The Journal of Immunology.* **206** (8), 1878–1889 (2021).
750 22 Schmid-Burgk, J. L. et al. Caspase-4 mediates noncanonical activation of the NLRP3
751 inflammasome in human myeloid cells. *European Journal of Immunology.* **45** (10), 2911–2917
752 (2015).
753 23 Baker, P. J. et al. NLRP3 inflammasome activation downstream of cytoplasmic LPS
754 recognition by both caspase-4 and caspase-5. *European Journal of Immunology.* **45** (10), 2918–
755 2926 (2015).
756 24 Cullen, S. P., Kearney, C. J., Clancy, D. M., Martin, S. J. Diverse activators of the NLRP3
757 inflammasome promote IL-1β secretion by triggering necrosis. *Cell Reports.* **11** (10), 1535–1548
758 (2015).
759 25 Sanders, M. G. et al. Single-cell imaging of inflammatory caspase dimerization reveals
760 differential recruitment to inflammasomes. *Cell Death & Disease.* **6**, e1813 (2015).
761 26 Charendoff, C. I., Bouchier-Hayes, L. Lighting up the pathways to caspase activation using
762 bimolecular fluorescence complementation. *Journal of Visualized Experiments: JoVE.* **133**, 57316,
763 (2018).
764 27 Bouchier-Hayes, L. et al. Characterization of cytoplasmic caspase-2 activation by induced
765 proximity. *Molecular Cell.* **35** (6), 830-840 (2009).
766 28 Riedhammer, C., Halbritter, D., Weissert, R. Peripheral blood mononuclear cells: Isolation,
767 Freezing, thawing, and culture. *Methods in Molecular Biology.* **1304**, 53–61 (2016).
768 29 Betsou, F., Gaignaux, A., Ammerlaan, W., Norris, P. J., Stone, M. Biospecimen science of
769 blood for peripheral blood mononuclear cell (PBMC) functional applications. *Current*
770 *Pathobiology Reports.* **7** (2), 17–27 (2019).
771 30 Perregaux, D. et al. IL-1 beta maturation: evidence that mature cytokine formation can be
772 induced specifically by nigericin. *The Journal of Immunology.* **149** (4), 1294–1303 (1992).
773 31 Cheneval, D. et al. Increased mature interleukin-1β (IL-1β) secretion from THP-1 cells
774 induced by nigericin is a result of activation of p45 IL-1β-converting enzyme processing. *Journal*
775 *of Biological Chemistry.* **273** (28), 17846–17851 (1998).
776 32 Fernandes-Alnemri, T. et al. The pyroptosome: a supramolecular assembly of ASC dimers
777 mediating inflammatory cell death via caspase-1 activation. *Cell Death and Differentiation.* **14** (9),
778 1590–1604 (2007).
779 33 Lu, A. et al. Unified polymerization mechanism for the assembly of ASC-dependent
780 inflammasomes. *Cell.* **156** (6), 1193–1206 (2014).
781 34 Muller-Eberhard, U., Javid, J., Liem, H. H., Hanstein, A., Hanna, M. Brief report: Plasma
782 concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases.
783 *Blood.* **32** (5), 811–815 (1968).
784 35 Shyu, Y. J., Liu, H., Deng, X., Hu, C. D. Identification of new fluorescent protein fragments
785 for bimolecular fluorescence complementation analysis under physiological conditions.
786 *Biotechniques.* **40** (1), 61–66 (2006).
787 36 Thornberry, N. A. et al. A novel heterodimeric cysteine protease is required for
788 interleukin-1βprocessing in monocytes. *Nature.* **356** (6372), 768–774 (1992).
789 37 Jensen, K., Anderson, J. A., Glass, E. J. Comparison of small interfering RNA (siRNA)
790 delivery into bovine monocyte-derived macrophages by transfection and electroporation.
791 *Veterinary Immunology and Immunopathology.* **158** (3–4), 224–232 (2014).
792 38 Tada, Y., Sakamoto, M., Fujimura, T. Efficient gene introduction into rice by

electroporation and analysis of transgenic plants: use of electroporation buffer lacking chloride ions. *Theoretical and Applied Genetics.* **80** (4), 475–480 (1990).

39 Bhowmik, P. et al. Targeted mutagenesis in wheat microspores using CRISPR/Cas9. *Scientific Reports.* **8** (1), 6502 (2018).

40 Sansom, D. M., Manzotti, C. N., Zheng, Y. What's the difference between CD80 and CD86? *Trends in Immunology.* **24** (6), 314–319 (2003).

41 Kurokawa, M., Kornbluth, S. Caspases and kinases in a death grip. *Cell.* **138** (5), 838–854 (2009).

Figure 1

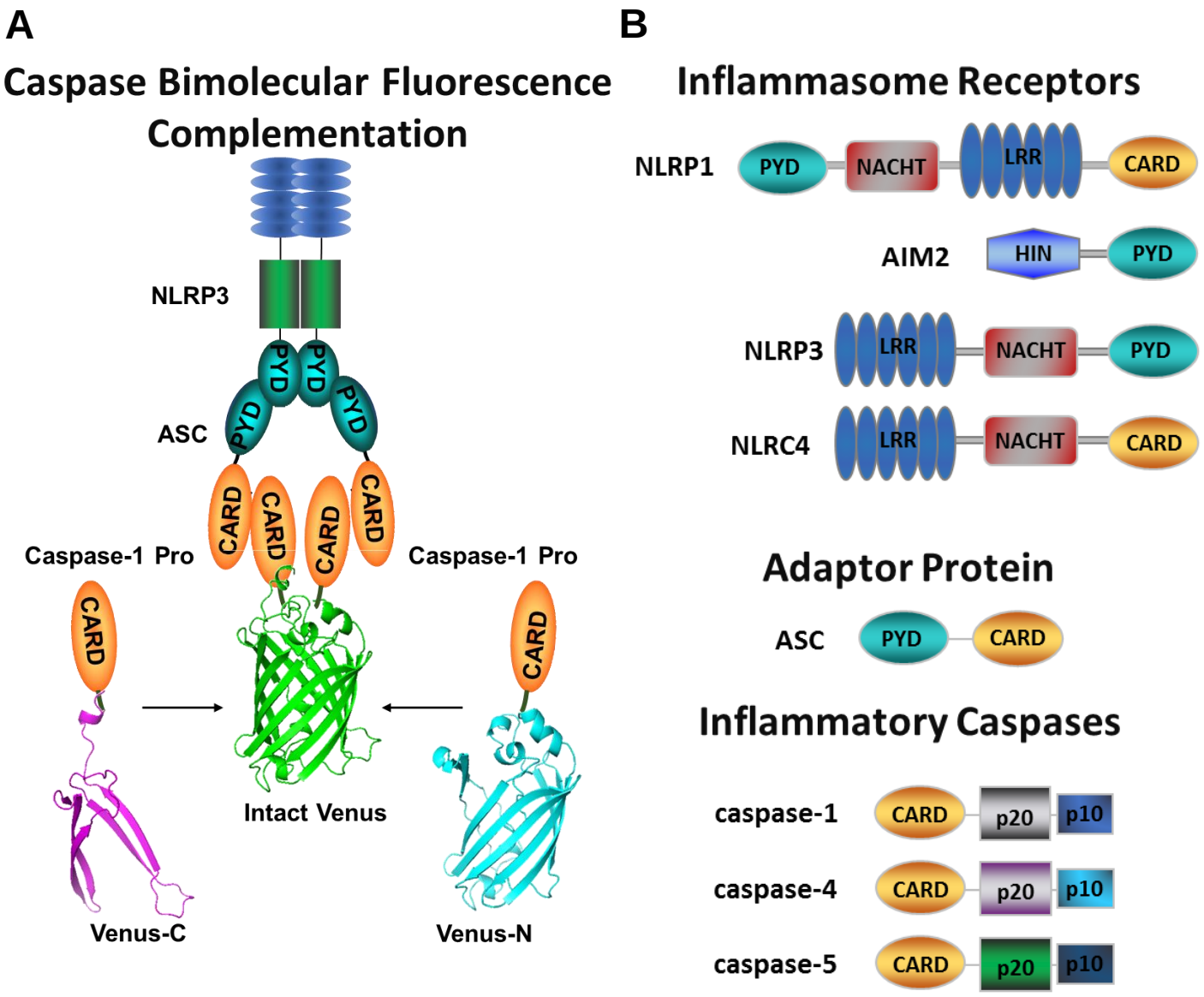


Figure 2

Figure 2

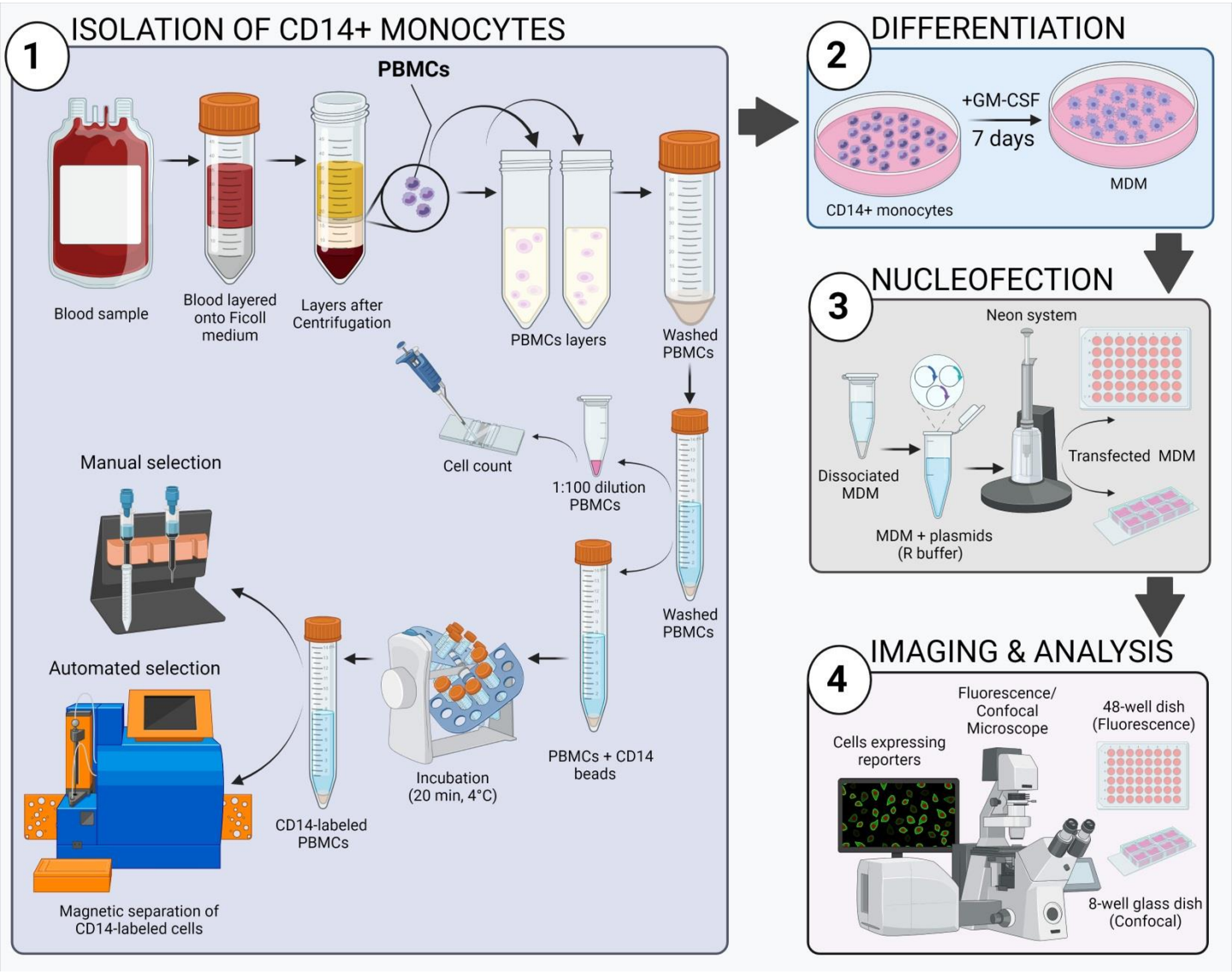


Figure 3

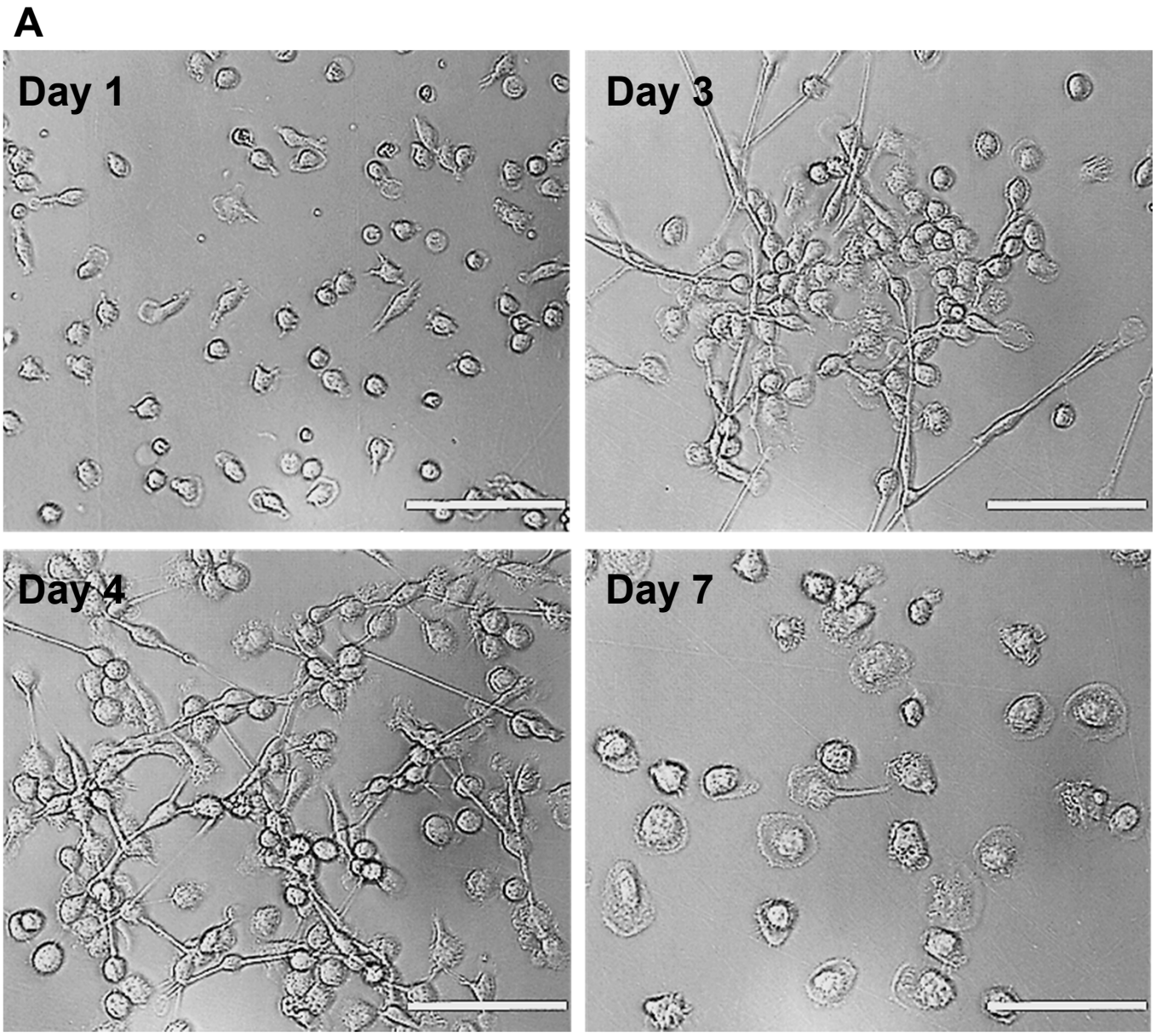
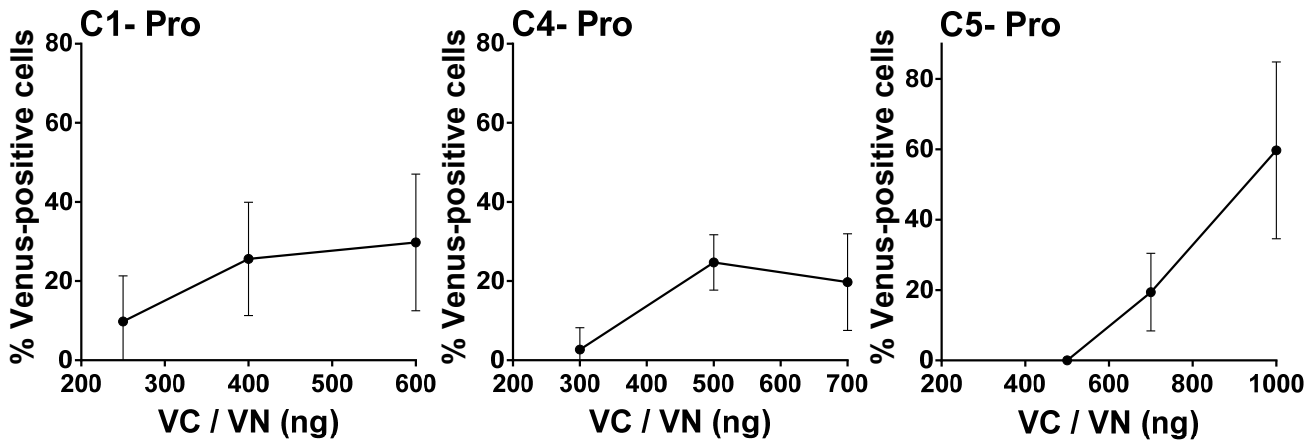
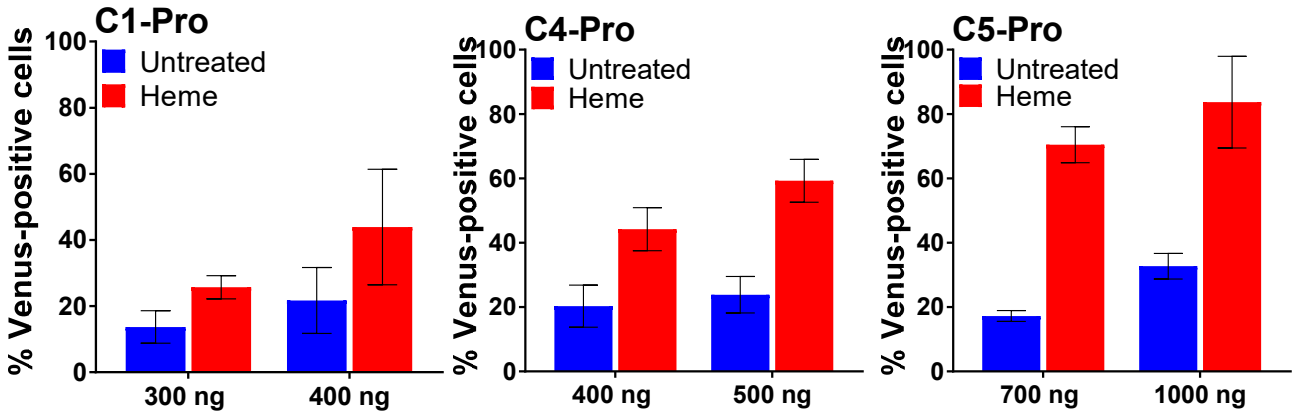


Figure 4

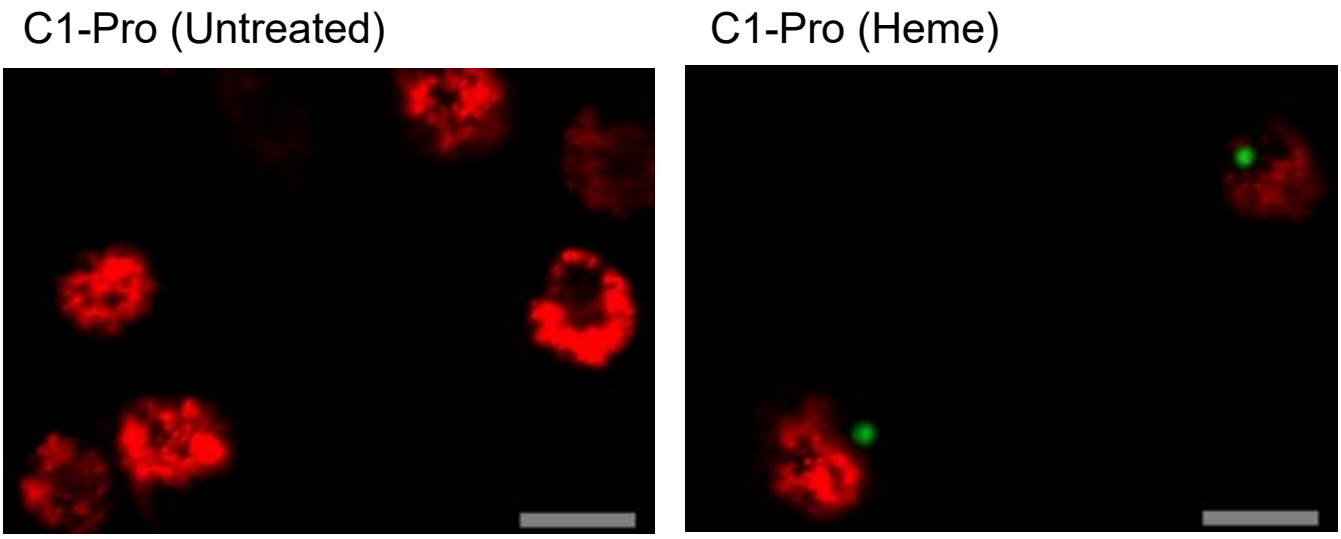
A



B



C



Response to Reviewers

General Remarks

We thank the reviewers for their insightful comments on the manuscript, and for highlighting that "the manuscript is well written and explains the method and background very well" and that "it will be useful for many researchers who are interested in studying the inflammasome in primary cells." We believe that our response to their suggestions, as delineated below, has resulted in a more clear and detailed protocol. We greatly appreciate the editor's and reviewers' time and effort that went into their suggestions to improve this protocol.

Detailed comments (Reviewers' comments are in italics; our responses are in plain text):

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Both authors have proofread the manuscript and have made pertinent corrections.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "The present protocol describes. …". Here the word limit is exceeding.

We have shortened the summary to 48 words following suggestions from editor and reviewers.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We revised the text and replaced personal pronouns appropriately.

4. Please include a citation (wherever appropriate) for the following lines: 85-88

Citations for lines 85-88 (Now lines 81-84) were included.

5.JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Gibco, sigma, Miltenyi Biotec, Thermo Fisher, etc.

We replaced any commercial language with generic terms in the manuscript and only included commercial details in the Table of Materials. We still use the term Neon as this protocol is adapted for a specific electroporation kit. Please advise if this needs to be changed.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee. Also, please mention whether the blood samples were collected from de-identified patients. If not, then please include the patient details and inclusion/exclusion criteria.

Ethics statement was included at the beginning of the protocol (lines 125-127) and step 1.1 specifies that anticoagulated blood is obtained from de-identified healthy individuals at a regional blood bank (lines134-135).

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Step 1.1: How were the blood samples obtained? If this step needs filming, please ensure patient availability at the time of filming.

Step 1.1 We obtain our blood samples from a regional blood bank that is from de-identified healthy donors (lines 134-135). We have removed mention of patient samples from the protocol steps except for clear instructions that IRB approval needs to be obtained if using them and we mention it in the discussion as an adaptation of the technique. Therefore, the procurement of the samples does not require filming.

Step 1.1.8: How was the aspiration done? Was a pipette used? What is the bleach concentration used? Please mention that the blood samples were handled following the institutional safety guidelines for human samples.

In lines 164-165, we now mention that a 10 mL pipette is used to aspirate the plasma and that this plasma is disposed into bleach (10%). In addition, we have added a note to the beginning of the protocol (lines 126-127) to indicate that blood samples were handled following the institutional safety guidelines for human samples.

Step 1.1.16: What do "a" and "d" stand for?

Acceleration (a) and deceleration (d) are now fully spelled out in step 1.1.16 (lines 196-197).

Step 1.1.17: This step is missing. Please check.

Numeral 1.1.17 was added by mistake and was deleted in this revised version.

Step 1.1.18: Is it MACS running buffer here again? Please specify.

The only MACS buffer used in this protocol is the "MACS running buffer." Therefore, this was corrected and was the term used throughout the paper.

Line 221: Please use µ instead of u.

The letter “u” was replaced with the Greek letter “µ” (now line 235) and where required.

Step 4.1, 4.2: Were the reporters and the plasmid diluted in the dilution buffer or any other solution was used?

Steps 4.1 and 4.2 now include that plasmids can be diluted in either nuclease free water or ½ X TE buffer (now lines 297-302).

Step 4.5: Please mention all the steps associated with this? We need action steps to show how this was done.

Step 4.5 was divided into steps 4.5 and 4.6 to include more details and action steps as suggested. The former step 4.6 is now step 4.8 (325-329) in this revised version.

Step 4.7: Please include all the button clicks, command lines, etc. in the software. We need actions to show how the software is used.

Step 4.7 now includes action steps to set up the transfection protocol on the Neon Device Screen.

Step 4.18: Please elaborate on the steps for using the fluorescence microscope providing all instrument settings and parameters.

Step 4.18 now includes sub items 4.18.1 – 4.18.4 to provide details on how to assess viability and transfection efficiency using an epifluorescence microscope.

*8. Figure 4A: Are the percentage cells over the background or above the background (is background % value taken as cut-off or is the percentage value calculated using the background). Please include both plus and minus error bars. Please indicate what *, **, *** stands for in the figure legends. What was the significance test used?*

As suggested by Reviewer 1, we have substituted the original Figure 4A with a plasmid titration experiment. This example illustrates how a preliminary plasmid titration can be performed using a positive control stimulus. This was not corrected for background levels. We have not included statistics as this new data is a representative experiment.
For the purpose of this protocol, we think this figure can illustrate better how this data is generally used and analyzed.
We have added both plus and minus error bars to all graphs and have defined the error bars in the figure legend as standard deviation of at least three independent counts per well.

9. Figure 4B: Please mention what the red and green colors stand for in the figure legend.

A description for each color is included now in the figure legend.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

In the revised manuscript we only include unpublished data and have not reused any figures.

11. As we are a methods journal, please ensure that the Discussion cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) Significance with respect to existing methods

e) Any future applications of the technique

We have restructured the discussion to include each of these sections.

12. Please do not abbreviate journal names in the References.

Full Journal names are now included in the References section.

Reviewer #1:

Major Concerns:

The authors provide enough precisions about their protocol. However, I think that the authors should illustrate 3 important illustration concerning their procedures including:

1-Experiments that illustrate the rate of differentiation of monocytes in macrophages

We thank the reviewer for this suggestion. We have updated Figure 3A to include images of CD14+ monocytes undergoing differentiation in GM-CSF at days 1, 3, 4 and 7.

2- The cell viability after transfection (90%, line 538)

In the revised version, we include specific steps to estimate cell viability using an epifluorescence microscope (4.18.1 – 4.18.3). In Figure 4C, we show representative confocal images obtained with a 20x objective of a field of cells 24 hours post-transfection, showing that the transfected cells are viable based on the appearance of the mitochondria (mitochondria in dead cells are highly fragmented) and the morphology of the cells (apoptotic cells are shrunken).

3-Titration experiment of the caspase BiFC plasmids as mentioned lines 542-546)

We have added examples of titration experiments in Figure 4. In Figure 4A, the percent Venus-positive transfected cells of three plasmid concentrations are shown for the three caspases (-1, -4, and -5) using an inflammatory stimulus, heme. In Figure 4B, two of the plasmid concentrations that showed a high response after treatment are used with and without treatment to determine BiFC-positivity and background levels, respectively.

In the manuscript, we can see several terms used for the cells used. Here the authors use human monocytes-derived macrophages (MDM) and it is this term that should be used. It is not human primary macrophages.

The term primary human macrophages was substituted for MDM throughout the manuscript.

In the abstract, the authors should add some precisions on the technique used and highlight the advantages/disadvantages to use this system.

We have reworded the abstract to include this information.

-Institutional guidelines and the approbation of a ethic committee should be mentioned for the use of blood from healthy of patients individuals. Did the authors precise if all anticoagulated blood tube could be used for this protocol?

Ethics statement was included at the beginning of the protocol (lines 125-126) and step 1.1 specifies that anticoagulated blood is obtained from de-identified healthy individuals at a regional blood bank (lines134-135). While this protocol is for blood from healthy donors, we also state that IRB approval is required to adapt it for patient samples.
We also indicated that blood samples for this protocol are obtained at a regional blood bank, where they are collected with citrate phosphate dextrose (CPD) solution. However, blood collected with other anticoagulants like sodium heparin, lithium heparin or EDTA can also be used for this protocol. We also included two references, so the reader can learn the pros and cons from using either source for this or other applications (lines 127-130).

The figure 3: it would be better to illustrate the differentiation of CD14+ monocytes into macrophages from day 1 to day 7.

Figure 3A now includes images of CD14+ monocytes undergoing differentiation in GM-CSF at days 1, 3, 4 and 7.

Minor Concerns:

-Line 22: ..."to obtain monocytes-derived macrophages from monocytes isolated from human blood samples"...

We have edited Line 22 as suggested.

-Line 23: BiFC must be written in full

The term BiFC was fully spelled out (now line 24).

-Line 24-25: ..." reporters into human MDMs"

Lines 24-25 were edited as suggested.

-Why the author precise "physiologically relevant" line 26

This sentence was unnecessary and therefore deleted.

-Line 38 ..."in MDm" and not primary macrophages

The term primary macrophages was substituted for MDM.

-Line 45: it is not differentiated macrophages but MDM, idem lines 127

The term primary macrophages was substituted again for MDM and throughout the manuscript.

-Line 49 it is not primary human macrophage, idem lines 118, 413, 427, 456, 463, 464, 477, 503, 587-588

The term primary human macrophages was substituted again for MDM and throughout the manuscript.

-In the first introduction the figure 1B (line 66) appears before figure 1 A (line 81) in the text

Figure 1A and 1B are swapped in the revised version, so they appear in the order in which they are mentioned. In addition, the text was modified to reflect this change.

-Line 90 "caspase-4 and -5"; there are the same mistakes lines 90-94-116-122-498-547 and 563

We respectfully disagree that this is a mistake as the caspases can be referred to by their full titles (e.g. caspase-4 and caspase-5) or shortened to caspase-4 and -5. We include the former for clarity in certain sections.

-Line 122 "caspase-11"

Caspase 11 was substituted for caspase-11 in the indicated line.

-Line 119: Can the authors precised the use of the term disease

We only use the term disease where the specific disease studied is up to the user – for example when we discuss further applications of the technique. This particular sentence has been changed to be more specific.

-Line 147: it is DPBS or PBS (see also table); the authors should be also used either 1X-PBS or X PBS

Either can be used. We changed this to 1X sterile PBS throughout because that is more accessible to most laboratories. We added a note to say that DPBS can be used.

-Check the supplier for the products mentioned for the first time (for example line 151 glutamax and P/S)

As requested by the editors, we have removed commercial language from the manuscript. This information is now included in the Table of Materials.

-Line 162: deleted (GE healthcare) as mentioned above

See above comment.

-It is MACS buffer or MACS running buffer?

In this revised version, MACS running buffer is the only term used throughout the manuscript.

-standardize in the text: min and minutes; h and hours; µL/uL/µl/ul

The units were standardized in the main text and figures as follows: h for hour; min for minutes; µL for microliter. This was corrected where applicable.

-Line 200: 1.1.17 ???

Thank you for catching this error, we have deleted 1.1.17 as it should not have been there.

-Line 201: Which buffer?

MACS running buffer was added to that line.

-Line 212: Can the authors precise the term mature mature?

The whole sentence was replaced by “see Figure 3A for appearance of CD14+ monocytes at various stages of differentiation in GM-CSF.”

-Line 247 trypsin 0.25% EDTA

The term trypsin-EDTA was replaced with the term trypsin-EDTA (0.25%) solution throughout the manuscript.

-Line 248: 5% CO2

We added the following to step 3.2 (now line 267): “incubate in a humidified 37 °C/5% CO_2 tissue culture incubator for 5 min.”

-Add supplier lines 219-230-247-345

Supplier information is only included in Table of Materials as per editorial request.

-Add space 200 µM and lines 461 µM, 10 µm; line 471

We have corrected these errors throughout the manuscript.

-Line 475 ... to obtain macrophages from monocytes isolated from human blood samples

We have added the following to the sentence (now lines 560-561): “from monocytes isolated from human blood samples”

-Add a reference to illustrate terms lines 571-573.

Citation for those lines is now included.

-Figure 2: In the differentiation box: note MDM below the right petri dish. In the nucleofection box: modified macrophage into MDM. The box 4 is imaging and analysis.

MDM is now used in the indicated boxes of Figure 2 and box 4 in Figure 2 is now labeled as IMAGING & ANALYSIS (see updated figure.)

-Table:

It is PBS -/- or +/+

In the Table of materials is now indicated “DPBS without calcium chloride and magnesium chloride.”

RPMI-1640.

In the Table of materials RPMI is now specified *RPMI 1640 Medium.*

Add 10 cm petri dish and mCherry

We have added both to the Table of Materials.

Reviewer #2:

Major Concerns:

-Are there any disadvantages/limitations to using this method? The only limitations discussed are related to other methods of introducing the reporter plasmids i.e. lentiviruses or the use of chemical transfection agents.

We have restructured the discussion to better highlight the limitations, troubleshooting and solutions of the BiFC technique in particular. This includes factors that contribute to high background levels or altered cellular behavior, factors that can cause false positive or false negative results (e.g. poor plasmid expression) and limitations of the technique with respect to how to interpret results as described in our answer to the comment below.

-Is it ever the case that these caspases are in close enough proximity to reform the fluorescent complex but that the endogenous caspases are not enzymatically active? If so, is there a way to determine if this is the case?

Yes, there is, and this is a limitation of the approach. Caspase BiFC measures the apical step required for caspase dimerization and activation, which is induced proximity but is does not directly measure dimerization or activation. This is a subtle yet important distinction. There are examples of posttranslational modifications that can inhibit dimerization of the catalytic domains, but not recruitment to the activation platform. We discuss this in detail and stress the importance of carrying out complementary functional studies to confirm any new finding using this technique.

-The introduction is very informative but is perhaps a bit long for this type of methods article. It could perhaps be shortened by moving some of the content to the discussion where relevant.

We thank the reviewer for this suggestion. We have edited the introduction for length and have moved some of the references to more technical parts to the discussion.

-Macrophages are notoriously difficult to detach from cell culture plates. What percentage of macrophages is detached using the trypsin-EDTA incubation described?

Normally, between 95 and 98% cells are detached at the end of the suggested trypsinization protocol. In step 3 of the protocol section, a note was included indicating the expected yield and suggesting performing two serial incubations with trypsin that do not exceed 5 min each to detach the cells without compromising their integrity. In addition, more details were included in the individual steps, which now include checking for cell detachment under a bright field microscope after trypsinization.

Minor Concerns:

-Step 4.8 The final sentence about not leaving cells for more than 15 min in R buffer seems critical and could be highlighted in a note.

This sentence is now included as a note in step 4, lines 294-295.