Chapter 9

Measuring NLR Oligomerization II: Detection of ASC Speck Formation by Confocal Microscopy and Immunofluorescence

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Abstract

Inflammasome assembly results in the formation of a large intracellular protein scaffold driven by the oligomerization of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC). Following inflammasome activation, ASC polymerizes to form a large singular structure termed the ASC "speck," which is crucial for recruitment of caspase-1 and its inflammatory activity. Hence, due to the considerably large size of these structures, ASC specks can be easily visualized by microscopy as a simple upstream readout for inflammasome activation. Here, we provide two detailed protocols for imaging ASC specks: by (1) live-cell imaging of monocyte/macrophage cell lines expressing a fluorescently tagged version of ASC and (2) immunofluorescence of endogenous ASC in cell lines and human immune cells. In addition, we outline a protocol for increasing the specificity of ASC antibodies for use in immunofluorescence.

Key words Inflammasome, ASC, Speck, Live-cell imaging, Immunofluorescence, Confocal microscopy, Flow cytometry

1 Introduction

The activation of certain innate immune receptors by microbial and endogenous danger signals leads to the formation of large multi-protein signaling platforms called inflammasomes. The assembly of inflammasomes mediates caspase-1-dependent proteolytic cleavage of important inflammatory mediators of the IL-1 family of cytokines, IL-1β, and IL-18, in concert with pyroptotic cell death [1]. In addition to their intracellular function, inflammasomes have recently been shown to display extracellular activity following their release from cells undergoing pyroptosis [2, 3]. Inflammasomes typically consist of three major components, the

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receptor/sensor (e.g., NLRP3, AIM2), the adaptor ASC, and the effector caspase-1. The association between sensor and ASC molecules is mediated via pyrin domain (PYD) interactions, while the involvement of caspase-1 is dependent on a caspase recruitment domain (CARD) present on both ASC and caspase-1. The activation of the inflammasome sensors triggers the recruitment and prion-like polymerization of ASC into large filamentous structures that triggers the inflammatory activity of caspase-1 [4, 5]. In resting cells, ASC displays soluble cytoplasmic and nuclear localization, but, upon assembly of inflammasomes, it is mobilized to form a large singular paranuclear ASC "speck" (~1 μm in diameter) [2, 6, 7]. Due to their relatively large size, ASC specks are easily visualized by various imaging techniques and therefore represent a convenient readout for inflammasome activation. We and others have previously described protocols for the generation of ASC fluorescently tagged cell lines using a retroviral transduction system and their use in high-throughput imaging for quantification and assessment of ASC speck formation following inflammasome activation [8, 9]; furthermore, methods to generate ASC specks from recombinant sources or purify from activated cells are also described [2, 7, 9].

In this chapter, we describe two imaging-based techniques for the detection of ASC specks. Firstly, we provide a protocol to assess the localization of ASC fluorescently tagged in live cells over time following NLRP3 inflammasome activation using confocal microscopy. Secondly, we present an optimized protocol for performing immunofluorescence (IF) of ASC in cell lines and primary human immune cells with commercially available antibodies. Finally, we provide a method for increasing the specificity of ASC antibodies to use in immunofluorescence by preclearance of anti-ASC antibodies using macrophages deficient in ASC (*Asc*$^{-/-}$ iMΦs).

2 Materials

2.1 Cells and Tissue Culture

1. THP-1 monocytes, or mouse-immortalized macrophages (iMΦs) expressing ASC fused to a fluorescent protein (e.g., cyan fluorescent protein, CFP)
2. THP-1 cells (American-type culture collection- ATCC), human monocyte cell line
3. Human peripheral blood mononucleated cells (PBMCs)
4. Wild-type bone marrow-derived macrophages (BMDMs)
5. ASC-deficient (*Asc*$^{-/-}$) BMDMs
6. Ficoll
7. Tissue culture flasks, 75 and 175 cm^2
8. 96-well tissue culture-treated plates for imaging (with flat glass bottom)

9. "Complete" Dulbecco's modified Eagle's medium (DMEM) cell culture medium: DMEM, supplemented with 10 % fetal bovine serum (FBS); optional: antibiotics (e.g., 10 μg/ml ciprofloxacin, or 100 U/mL of Penicillin-Streptomycin)
10. "Complete" Roswell Park Memorial Institute (RPMI) cell culture medium: RPMI supplemented with 10 % FBS; optional: antibiotics (e.g., 10 μg/ml ciprofloxacin, or Penicillin-Streptomycin)
11. Phosphate-buffered saline solution (PBS): 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, and 2 mM KH_2PO_4 and pH 7.4
12. Mammalian cell culture facility with an incubator maintained at 37 °C and 5 % CO_2 and 95 % air

2.2 Stimuli

1. Phorbol 12-myristate 13-acetate (PMA)
2. Ultrapure lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS)
3. Nigericin
4. Adenosine 5′-triphosphate disodium salt hydrate (ATP) (*see* **Note 1**)

2.3 Immunostaining and Flow Cytometry

1. Sixteen percent formaldehyde (store protected from light)
2. Fixation buffer: 4 % formaldehyde in PBS (*see* **Note 2**)
3. Permeabilization and blocking (perm/block) buffer: 10 % goat serum, 1 % FBS, and 0.5 % Triton-X100 in PBS
4. Wash buffer: PBS
5. DRAQ5 (store at 4 °C protected from light; DRAQ5 is toxic, so handle with care)
6. Recombinant cholera toxin subunit B-conjugated with Alexa Fluor 555® (this product is exclusive of Life Technologies, although other fluorochrome conjugates from other companies are also suitable). Fluorochrome-conjugated Weat Germ Agglutinin also work well and can be used as an alternative cell membrane staining.

2.4 Antibodies and Conjugates

1. There are different anti-ASC antibodies suitable to detect ASC; here we use the rabbit polyclonals (pAb) anti-ASC from Adipogen (clone AL177) and Santa Cruz (Clone N-15-R) and the mouse monoclonals (mAbs) anti-ASC from Millipore (clone 2EI-7), and BioLegend (purified TMS-1 antibody, clone HASC-71), although other clones might be also suitable.
2. Mouse-purified IgG isotype control antibody
3. Rabbit-purified IgG isotype control antibody

4. Goat anti-rabbit-Alexa Fluor 488® and anti-mouse-Alexa Fluor 488® secondary antibodies (these antibodies are exclusive of Life Technologies, although other fluorochrome conjugated antibodies from other companies are also suitable)

2.5 Imaging and Flow Cytometry

1. Epifluorescence microscope with a filter for CFP (e.g., 458 nm laser with 510/80 nm emission optics) and a filter for DRAQ5 (e.g., Filter Set 50 from Zeiss or similar: excitation band pass 640/30, dichroic mirror FT 660, emission band pass 690/50) and with 20× and 63× objective. As an alternative, a confocal microscope with laser lines 458 nm (or 405 nm) and 647 nm (or 633 nm) and appropriate filters (as above) can be used.
2. Image analysis software such as Volocity 6.01 or ImageJ (available as freeware from http://rsbweb.nih.gov/ij).
3. Flow cytometer equipped with argon laser and filter settings for CFP (e.g., 445 nm laser with 510/80 nm emission optics) and GFP/FITC (e.g., 488 nm laser with 505/45 nm emission optics).
4. Flow cytometry software such as FlowJo V9.8 or later.

3 Methods

3.1 Live Imaging of ASC Speck Formation

This method is suitable to study ASC speck formation by live imaging upon NLRP3 inflammasome activation in cells stably expressing fluorescent ASC.

3.1.1 Stimulation and Counterstaining for Live Imaging

1. **Optional**: To minimize cell lost during the staining protocol, it is recommended to coat the wells of 96-well imaging plates with either poly-L-lysine or mouse collagen IV before seeding the cells (*see* **Note 3**).
2. Seed 0.5×10^5 iMΦs in complete DMEM or 1×10^5 THP-1 monocytes in complete RPMI (supplemented with 100 nM of PMA) per well of a 96-well imaging plate (*see* **Note 4**).
3. Incubate the cells overnight at 37 °C and 5 % CO_2.
4. The next day, prime cells with 200–250 ng/ml of LPS for iMΦs or 1 μg/ml of LPS for THP-1 for 2–3 h in 100 μl of complete media (use DMEM for iMΦs and RPMI for THP-1).
5. If nuclei and/or membrane staining is desired, it can be done at this stage. During the last 30 min of LPS priming, DRAQ5 or cholera toxin subunit B (CtxB) can be added to the cells. *See* **Note 5**.
6. Place the plate containing the cells in the imaging stage in the microscope and adjust the laser intensities, the focal plane, and the desired field for imaging. *See* **Note 6**.

7. Remove the media containing LPS, and replace it with 100 μl of fresh complete media containing NLRP3 inflammasome activators (10 μM of nigericin or 5 mM ATP).
8. Place the plate in a confocal microscope equipped with an incubation chamber set at 37 °C and 5 % CO_2 and start imaging (*see* **Note 7**).

3.1.2 Confocal Settings and Image Acquisition

9. Use the 20× or 63× objective with filters for the fluorescent tag of your ASC construct (i.e., we use a laser with 458 nm [or 405 nm] wavelength to excite ASC-CFP, 561 nm to excite CtxB-Alexa 555, and one with 647 nm [or 633 nm] to excite DRAQ5).
10. During acquisition, make sure to use the same imaging parameters (gain, light intensity, pinhole, etc.) for all conditions.
11. The formation of ASC specks is rapid, so adjust the interval between every picture accordingly. We recommend following the assembly of ASC fluorescent protein by acquiring images every 5 min for up to 90 min total imaging time.
12. While imaging the fluorescently tagged version of ASC, you should see a dramatic change in its cellular distribution. The fluorescent ASC changes from a weak diffuse signal present throughout the cell (including the nucleus) to a singular bright spot in an activated cell (Fig. 1a).

3.2 Immunofluorescence of ASC Specks with Antibodies

ASC specks can also be targeted by antibodies immediately upon inflammasome activation [2] (*see* **Note 7** and Fig. 1b).

We have tested several commercially available anti-ASC antibodies, and their combination with secondary antibodies directly conjugated to Alexa 488, and found significant differences in their performance (e.g., signal/noise and specificity) (*see* Fig 1b, **Note 8–9,** and Fig. 2). Irrespective of the anti-ASC antibody of your choice, we provide here a general protocol to stain endogenous ASC in inflammasome-activated cells:

1. *Optional*: To minimize cell lost during the staining protocol, it is recommended to coat the wells of 96-well imaging plates with either poly-L-lysine or mouse collagen IV before seeding the cells (*see* **Note 3**).
2. Seed wild-type THP-1 monocytes, iMΦs, or BMDMs in 96-well confocal plates, prime cells with LPS, and activate the NLRP3 inflammasome with nigericin or ATP as described in the previous section.
3. Upon activation (60 min for ATP and 90 min for nigericin), carefully remove the media on the cells and slowly add 100 μl of the fixation buffer and incubate samples for 30 min at 37 °C (*see* **Note 10**).

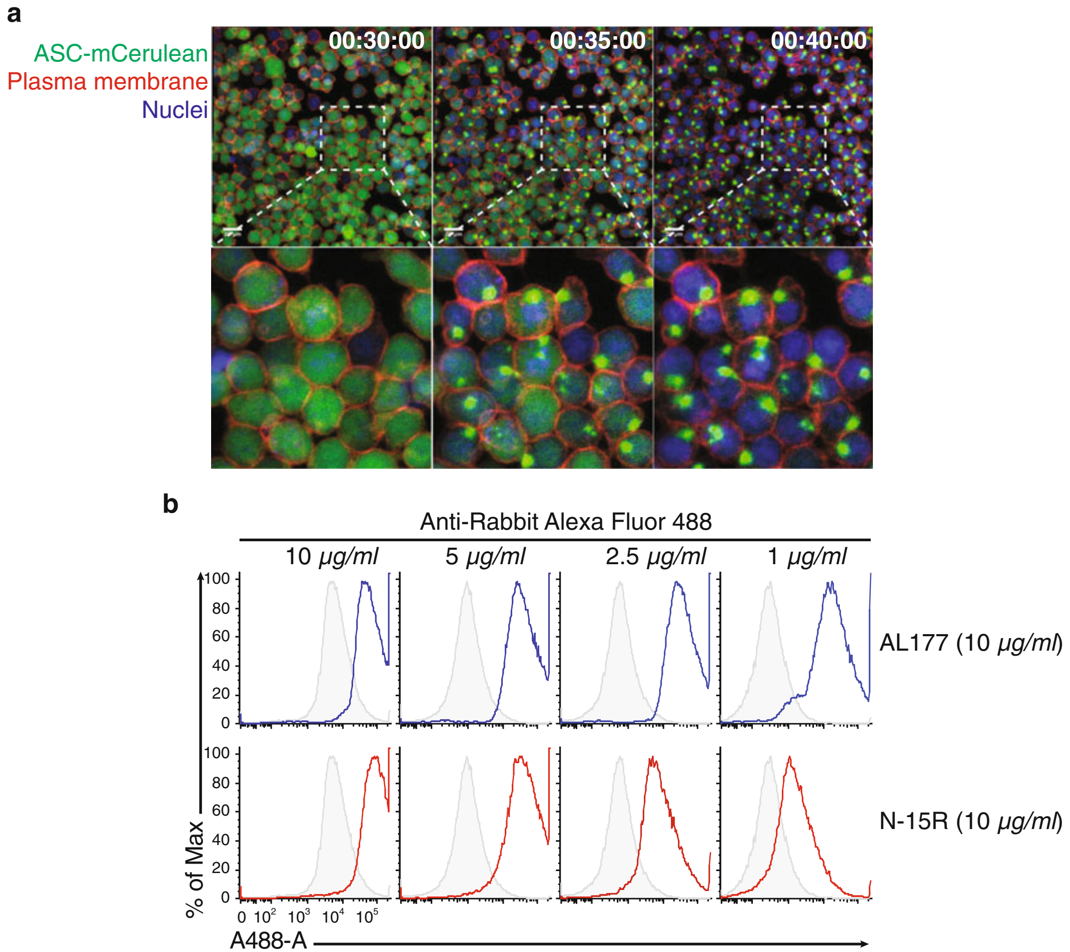

Fig. 1 Life imaging of ASC Specks formation in ASC-mCerulean expressing cells (**a**) Typical result of unstimulated and LPS-primed, nigericin-activated ASC-mCerulean-expressing human THP-1 monocytes. Images were acquired in a Leica TCS SMD FLCS confocal microscope (63× objective). Maximum projection images are shown. Note the redistribution of ASC-mCerulean from a broad and dim fluorescence in the *left panels* to a bright, small speck-like fluorescence in the *right panels*. Nuclei were stained with DRAQ5 (5 μM) and plasma membrane with cholera toxin B-Alexa Fluor 555 (5 μg/mL). *White numbers top-right corners* are imaging time. (**b**) Titration of the secondary anti-species IgG specific antibodies directly conjugated to Alexa Fluor 488 dye for detection of ASC specks by Flow Cytometry

4. Carefully wash the cells twice by slowly adding 100 μl PBS (*see* **Note 11**) to remove fixation buffer.
5. Slowly add 100 μl of the perm/block buffer to the cells and incubate for 30 min at 37 °C. This blocking step is important to reduce unspecific staining.
6. Slowly remove the perm/block buffer and replace it with 100 μl/well of fresh perm/block buffer containing 1 μg of the anti-ASC of your choice (*see* **Note 9** and Fig. 2).

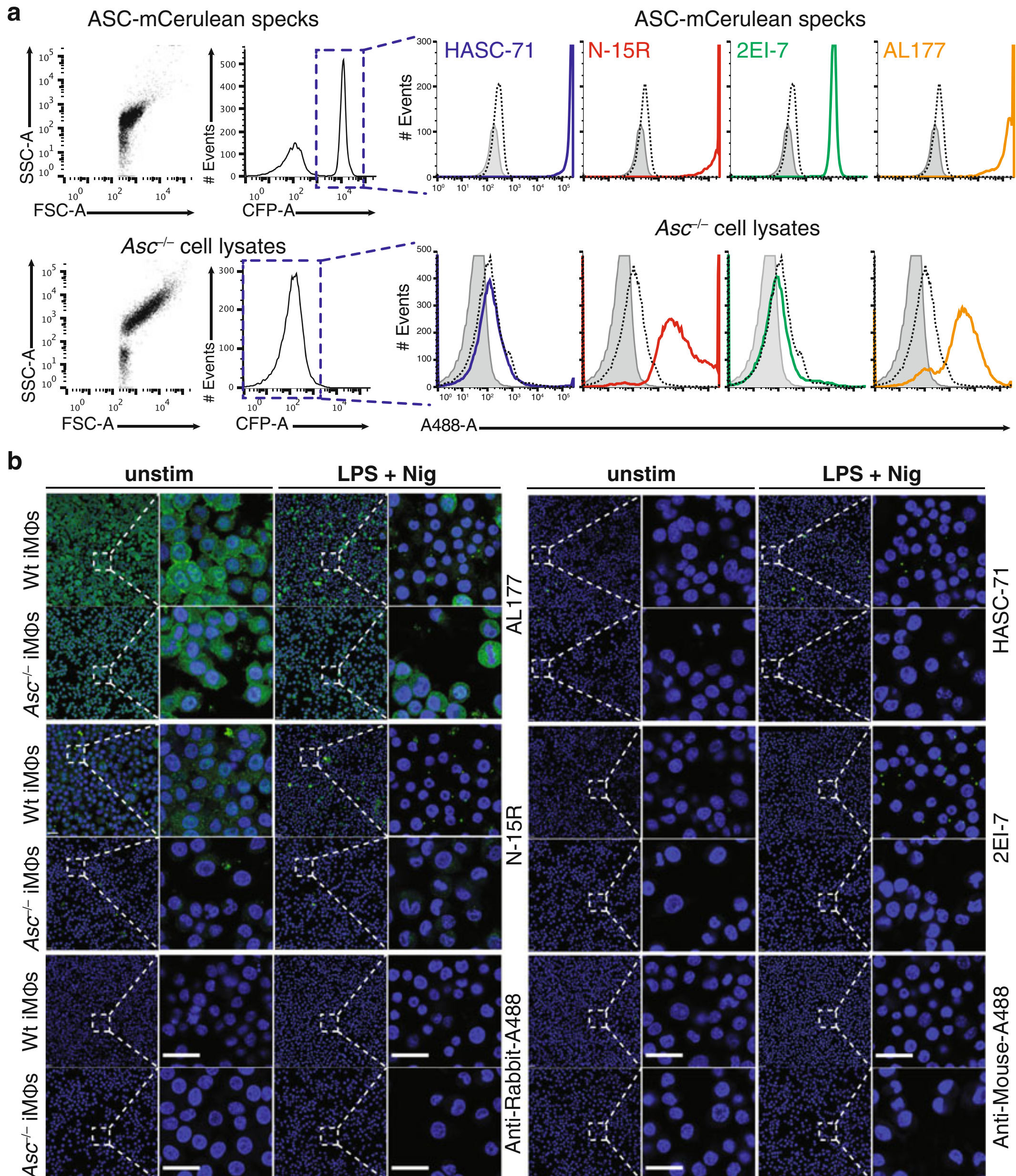

Fig. 2 Optimization of ASC speck staining for immunofluorescence and confocal microscopy (**a**) Flow cytometric analysis of ASC-mCerulean specks, or lysates of *Asc*$^{-/-}$ iMΦs, that were stained with a series of commercially available anti-ASC antibodies, followed by staining with 2.5 μg/ml of secondary antibodies directly conjugated to Alexa 488. Note that although all antibodies tested yielded a distinct staining of ASC-mCerulean specks, the antibodies from Santa Cruz clone N15-R (*red trace*) and Adipogen (*orange trace*) also reacted quite highly with *Asc*$^{-/-}$ iMΦs. (**b**) Confocal imaging of resting or LPS-primed, nigericin-activated wild-type or *Asc*$^{-/-}$ iMΦs. Cells were fixed and stained with a series of commercially available anti-ASC antibodies, followed by staining with secondary antibodies directly conjugated to Alexa 488. As a control cells were incubated with the secondary antibodies alone

7. Incubate cells at room temperature for at least 1 h (*see* **Note 12**).
8. Wash cells gently three times with 200 μl of block/perm buffer (*see* **Note 11**).
9. Stain cells with secondary antibody (2.5 μg/ml, Fig. 1b) in 100 μl of block/perm buffer and incubate for 1 h at RT.
10. Wash cells gently three times with 200 μl block/perm buffer.
11. If nuclear or plasma membrane staining is desired, add 5 μM of DRAQ5 and/or 5 μg/ml of CtxB in 200 μl PBS and incubate 5–10 min at 37 °C.
12. Wash the cells gently three times with 200 μl PBS (*see* **Note 11**).
13. Plates can be stored at 4 °C, with 100 μl of PBS per well until imaged.
14. Image cells with a fluorescent or confocal microscope, as described above in Subheading 3.1.2.
15. Similar to live imaging of fluorescent ASC-expressing cells, you should see a dramatic change in the distribution of antibody-targeted ASC within the cell (Fig. 3).

3.3 Imaging of ASC Specks Using Antibodies in PBMCs

1. Isolate human PBMCs using Ficoll according to manufacturer's instructions.
2. PBMCs adhere very weakly to confocal plates. To minimize cell lost during the staining protocol, it is recommended to coat the wells of 96-well imaging plates with either poly-L-lysine or mouse collagen IV before seeding the cells (*see* **Note 3**).
3. Seed 1×10^5 cells/well in a 96-well glass-bottom plate (*see* **Note 4**).
4. Let the cells adhere for at least 3 h (or overnight) at 37 °C.
5. Gently remove the media and wash the cells once with PBS.
6. Prime the cells with 200–1000 pg/ml LPS for 2–3 h in 100 μl of complete DMEM per well (*see* **Note 13**).
7. Remove the supernatants and wash the cells once with 100 μl of PBS to remove serum-containing medium.
8. Add 100 μl/well of serum-free medium containing 10 μM of nigericin or 5 mM of ATP and incubate cells at 37 °C, 5 % CO_2 for 30–60 min with ATP or 90 min with nigericin.
9. After inflammasome activation, centrifuge the plate at $400 \times g$ for 5 min to pellet floating cells and carefully remove the supernatants.
10. Fix cells with 100 μl of fixation buffer/well and incubate at 37 °C for 30 min (or overnight at 4 °C).
11. Wash the cells very carefully three times with PBS and incubate at 37 °C for 5 min between washes (*see* **Note 11**).

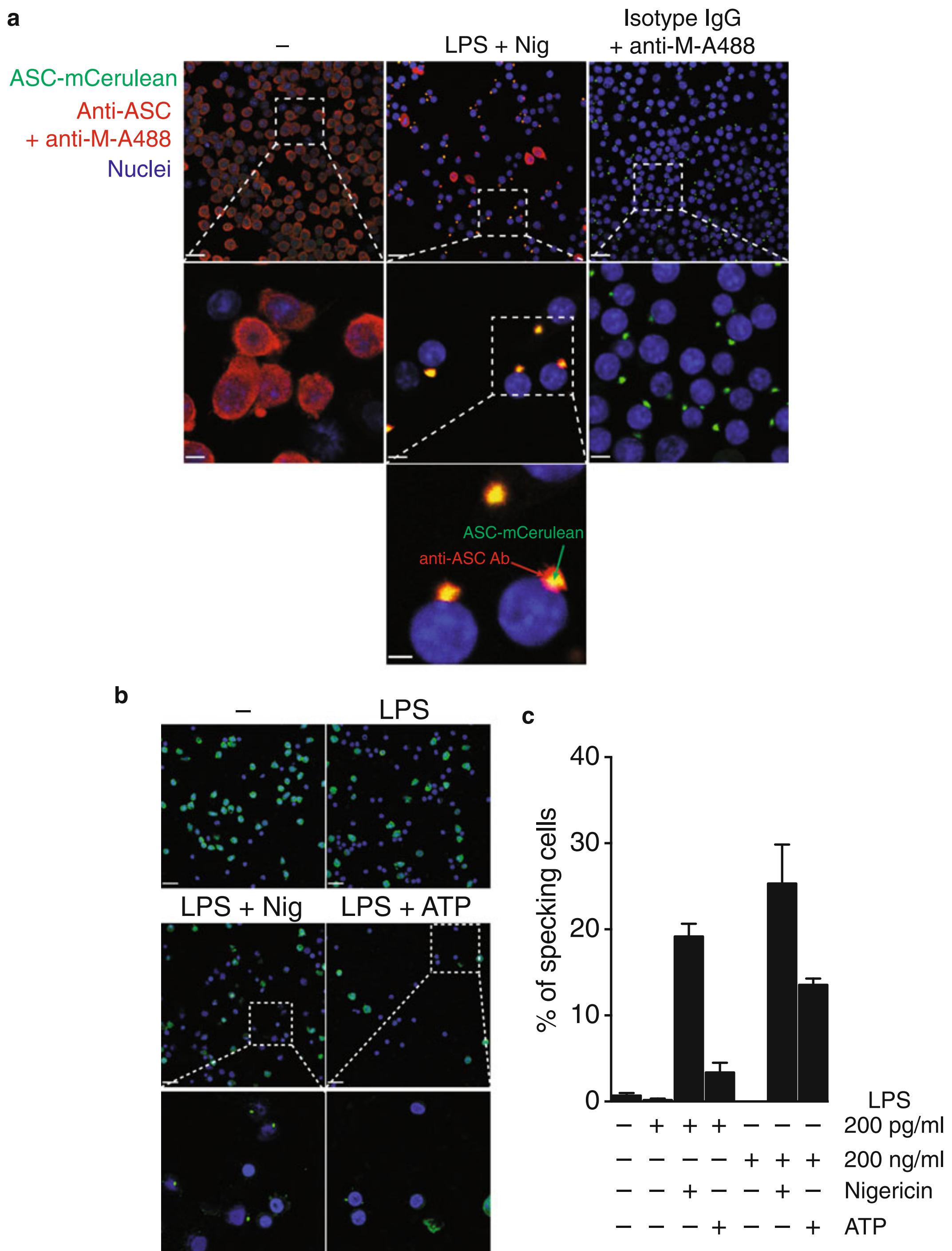

Fig. 3 Immunofluorescence of ASC speck in mouse iMΦs and human primary cells (**a**) Confocal imaging of resting or LPS-primed nigericin-activated ASC-mCerulean-expressing iMΦs. Cells were fixed and stained with anti-ASC (HASC-71, BioLegend) Abs, followed by staining with secondary anti-mouse IgG directly conjugated to Alexa Fluor 488 dye. As a control, cells were incubated with a mouse-purified IgG isotype, followed by staining with secondary antibody. Nuclei were stained with DRAQ5. (**b**) Confocal imaging of resting (–) or LPS-primed (LPS) human PBMCs that were either left untreated or activated with nigericin (LPS + Nig) or ATP (LPS + ATP). Cells were fixed and stained with anti-ASC (HASC-71, BioLegend) antibody, followed by staining with 2.5 μg/ml of anti-rabbit IgG directly conjugated to Alexa 488. (**c**) Quantification of ASC specks in human PBMCs treated and stained as in (**b**)

12. Incubate the cells with 100 µl of perm/block buffer for 30 min at 37 °C.
13. Add 100 µl of perm/block buffer containing 10 µg/ml of the anti-ASC of your choice in the desired wells and incubate plates at room temp for 1 h or overnight at 4 °C.
14. Carefully wash the wells three times with PBS.
15. Add 100 µl of perm/block buffer containing 2.5 µg/ml of anti-rabbit IgG Alexa Fluor 488 per well and incubate the plate at 37 °C for 40–60 min.
16. Carefully wash the wells three times with PBS.
17. If nuclear or plasma membrane staining is desired, add DRAQ5 and/or CtxB as described above.
18. Carefully wash the wells three times with PBS.
19. Add 100 µl of PBS to the cells and proceed to imaging.
20. Image cells by confocal microscopy as described above in Subheading 3.1.2.

3.4 Protocol to Increase the Specificity of Anti-ASC Antibodies for Immunofluorescence

Antibodies subjected to this method can be analyzed by flow cytometry to assess their binding capacity to purified ASC specks or *Asc*$^{-/-}$ cell lysates. As shown in Fig. 4, the Anti-ASC N-15R antibody (Santa Cruz) stains ASC-mCerulean specks; however, it also reacts with *Asc*$^{-/-}$ cell lysates (mock specks) yielding high staining background. However, such a nonspecific signal is not observed with the anti-ASC HASC-71 (BioLegend) antibody. It is thus possible to "clean" the anti-ASC N-15R antibody to significantly reduce nonspecific staining in *Asc*$^{-/-}$ cell lysates, without affecting the staining of ASC specks. This method may also be applied to other anti-ASC antibodies. Here we provide a protocol to "clean" anti-ASC antibodies:

1. Harvest ~1×10^6 *Asc*$^{-/-}$ cells/ml and transfer the cell suspension to 50 ml tubes.
2. Wash cells twice with cold PBS by pelleting them at 400 ×*g* for 5 min at 4 °C.
3. Resuspend cells in 10 ml of block/perm buffer and incubate them for 30 min at room temperature.
4. Wash cells twice by pelleting them at 400 ×*g* for 5 min at 4 °C and resuspending in PBS.
5. Resuspend cells in 1 ml of block/perm buffer containing 20 µg of Anti-ASC N-15R antibody.
6. Incubate cells overnight at 4 °C with rotation, to allow the antibody to evenly diffuse.
7. The next day, pellet the cells at 400 ×*g* for 5 min and transfer the supernatants containing unbound antibodies to a new tube.

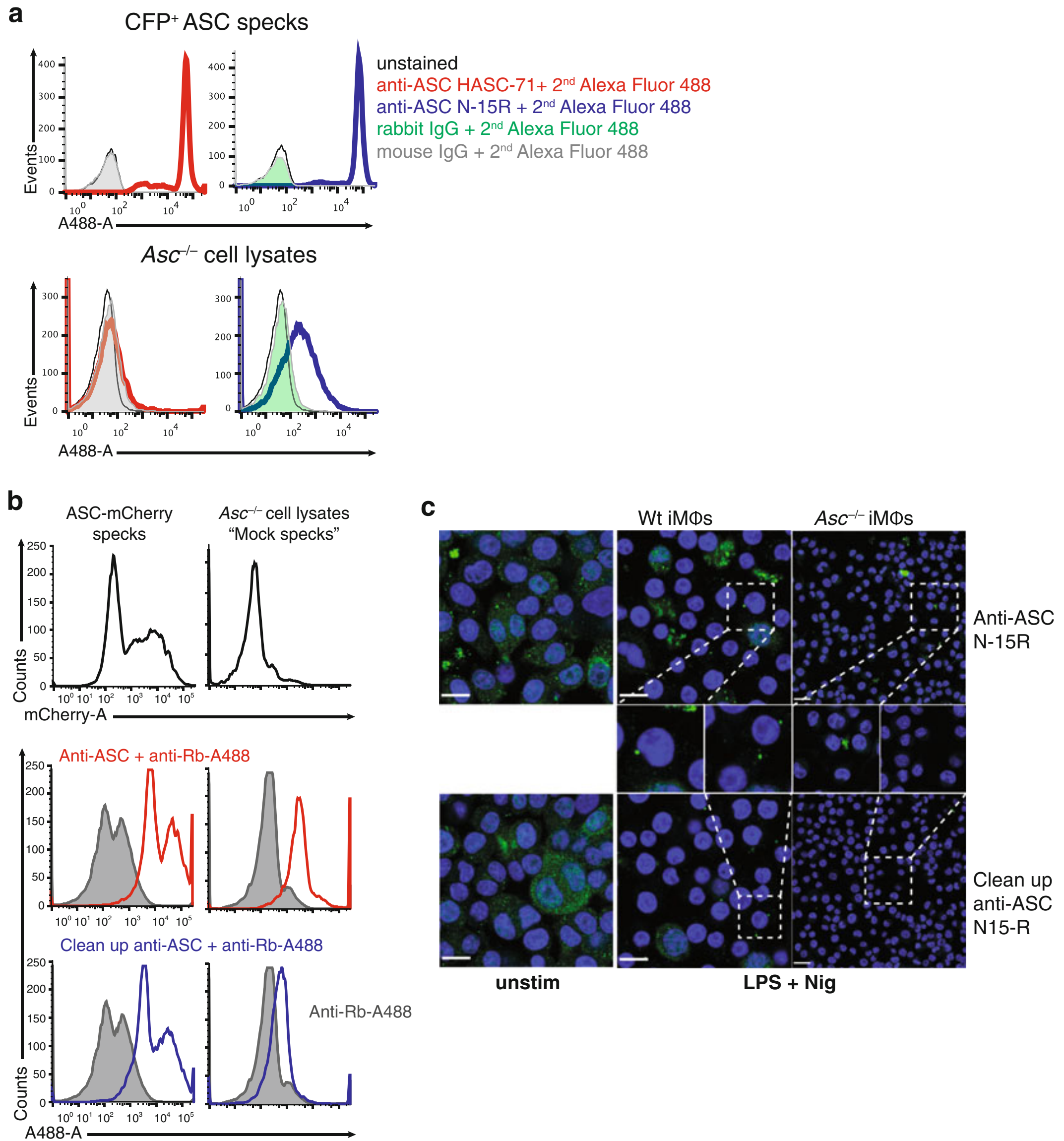

Fig. 4 Optimization of anti-ASC performance for immunofluorescence. (**a**) Flow cytometry of ASC-mCerulean specks or lysates of *Asc*−/− iMΦs stained with anti-ASC HASC-71 (BioLegend, *red trace*) or N-15R (Santa Cruz, *blue trace*), followed by staining with 2.5 μg/ml of anti-mouse or anti-rabbit IgG directly conjugated to Alexa Fluor 488 dye. As a control, cells were incubated with a mouse- or rabbit-purified IgG isotype, followed by staining with secondary antibody. Note that although both antibodies yield a staining of ASC-mCerulean specks, the Santa Cruz antibody also reacts quite highly with *Asc*−/− iMΦs. (**b**) Flow cytometry analysis as in a of ASC-mCerulean specks or lysates of *Asc*−/− iMΦs stained with anti-ASC from Santa Cruz before (*red trace*) of after (*blue trace*) cleanup of this antibody, followed by staining with 2.5 μg/ml of anti-rabbit IgG directly conjugated to Alexa 488. (**c**) The comparison of the anti-ASC Santa Cruz abs in resting (unstim) or LPS-primed, nigericin-activated (LPS + Nig) wild-type iMΦs before and after cleanup. Cells were fixed and stained with anti-ASC antibodies, followed by staining with 2.5 μg/ml of anti-rabbit IgG directly conjugated to Alexa 488

8. Filter the supernatants at 0.22 μm to remove any cell debris.
9. Determine the protein concentration in the supernatants (e.g., by BCA or Bradford).
10. Test the clean anti-ASC antibody in flow cytometry or confocal assays (Fig. 4).

4 Notes

1. Many inflammasome stimuli are toxic and need to be handled with care. Follow the manufacturer's instructions. To activate NLRP3, the following conditions can be used: prime cells with 200 ng/ml LPS for 2–3 h, and then stimulate with 5 mM ATP for 30–60 min or 10 μM nigericin for 60–90 min.
2. Formaldehyde is toxic and carcinogenic. Wear protective equipment and dispose of formaldehyde according to the local regulations. Formaldehyde solutions need to be made up fresh every time, as formaldehyde oxidizes to formic acid over time.
3. As inflammasome activation leads to pyroptotic cell death, cells often detach from the plate and can therefore be washed away during the washing steps, leaving very few cells for imaging. This can be minimized by pre-coating the plates with either 30–40 μl/well of poly-L-lysine for 1 h, or with 1–5 μg/ml of mouse collagen IV for 30 min at 37 °C. Wash the plates with sterile injection water and let them dry completely before seeding the cells.
4. It is advised to not use the outer wells of the 96-well plate, as they are more prone to evaporation. Thus, only use the middle 60 wells from columns 2 to 10 and from rows B to G.
5. For live imaging, DRAQ5 and/or cholera toxin B (CtxB) can be added to the cells during the final 30 min of LPS priming. Dilute DRAQ5 or CtxB in PBS and add to the cells at a concentration of 2.5–5.0 μM for DRAQ5 and 5 μg/ml for CtxB. Incubate for 5–10 min at 37 °C. Wash it once with 100 μl/well of PBS and replace the PBS with fresh medium. Follow with inflammasome stimulation.
6. Once the inflammasome stimuli are added, cells are rapidly activated. Therefore, it is recommended for optimal imaging performance to pre-image the plates before activation. This can be done during the last 30 min of the LPS priming step. Place the plate containing the cells in the imaging stage in the microscope and adjust the laser intensities, the focal plane, and the desired field for imaging. If stacks in the *z*-axis are desired, this can also be adjusted at this point. This procedure will spare the time needed to set up the microscope and guarantee that the cells are imaged from the very initial stages of activation.

7. Inflammasome activation in cells usually results in disintegration of the plasma membrane and cell morphology. Cells will often blow and round up. It is thus recommended to take z-stacks during imaging and display the maximum intensity projection of these z-stacks. Cells with specks will often round up, and some of the specks will be located further away from the focal plane.
8. To optimize staining of ASC specks for immunofluorescence, it is important to titrate the antibodies to get the best signal/noise ratio. We found out that most primary anti-ASC antibodies work well at a concentration of 10 μg/ml, thought lower concentrations also work. We have tested different concentrations of the fluorescently conjugated secondary antibodies and found that the optimal signal-to-noise ratio was obtained when secondary antibodies were used at a final concentration of 2.5 μg/ml (Fig. 1b). Unless stated otherwise, this concentration was used throughout in the experiments described in this book chapter.
9. We have tested most of the commercially available anti-ASC antibodies by flow cytometry and confocal imaging (Fig. 2), in different concentrations and conditions (not shown). In our laboratory conditions, the mouse monoclonal anti-ASC from BioLegend and Millipore displayed the best signal/noise ratio and were highly specific, as they did not react with *Asc*$^{-/-}$ cell lysates (Fig. 2). Although all antibodies tested yielded a distinct staining of ASC specks, the antibodies from Santa Cruz and Adipogen displayed less specificity as they reacted quite highly with *Asc*$^{-/-}$ iMΦs (Fig. 2a, traces of red and orange for Santa Cruz and Adipogen antibodies, respectively).
10. Is it recommended to keep all buffers (fixation and block/perm) cold.
11. It is crucial at this point to wash the wells very gently to avoid loss of cells from the plate.
12. Incubation with anti-ASC antibodies overnight at 4 °C usually yields better signal.
13. Human PBMCs can be primed with 200 pg/ml or 1 ng/ml of LPS. In our hands, 1 ng/ml resulted in a higher percentage of inflammasome-activated cells (as assessed by quantifying the cells that contains ASC specks, Fig. 3c).

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