Video Article

Study of Phagolysosome Biogenesis in Live Macrophages

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Abstract

Phagocytic cells play a major role in the innate immune system by removing and eliminating invading microorganisms in their phagosomes. Phagosome maturation is the complex and tightly regulated process during which a nascent phagosome undergoes drastic transformation through well-orchestrated interactions with various cellular organelles and compartments in the cytoplasm. This process, which is essential for the physiological function of phagocytic cells by endowing phagosomes with their lytic and bactericidal properties, culminates in fusion of phagosomes with lysosomes and biogenesis of phagolysosomes which is considered to be the last and critical stage of maturation for phagosomes. In this report, we describe a live cell imaging based method for qualitative and quantitative analysis of the dynamic process of lysosome to phagosome content delivery, which is a hallmark of phagolysosome biogenesis. This approach uses IgG-coated microbeads as a model for phagocytosis and fluorophore-conjugated dextran molecules as a luminal lysosomal cargo probe, in order to follow the dynamic delivery of lysosmal content to the phagosomes in real time in live macrophages using time-lapse imaging and confocal laser scanning microscopy. Here we describe in detail the background, the preparation steps and the step-by-step experimental setup to enable easy and precise deployment of this method in other labs. Our described method is simple, robust, and most importantly, can be easily adapted to study phagosomal interactions and maturation in different systems and under various experimental settings such as use of various phagocytic cells types, loss-of-function experiments, different probes, and phagocytic particles.

Video Link

The video component of this article can be found at https://www.jove.com/video/51201/

Introduction

Professional phagocytes, including macrophages, play a critical in the immune system. In addition to being the first line of defense in the innate immune system, they also play a critical role in activation of the adaptive immunity through their signaling and antigen presenting role[1-3]. While the function of professional phagocytes is multifaceted, phagosome maturation is the critical backbone of the bactericidal and antigen processing function of the professional phagocytes[4,5]. Upon the receptor mediated engulfment and uptake of a phagocytic target, such as bacteria, the nascent phagosome goes through a complex and well-orchestrated sequence of interaction and exchanges with compartments of the endocytic network and several other cellular organelles[6]. The nature of the compounds exchanged with these cellular entities as well as the regulation and the timing of fusion events determines the luminal phagosomal milieu and the phagosomal membrane composition and, thus[7], the fate of the maturing phagosome[8].

The physiological relevance of phagosome maturation is exemplified by the diverse strategies employed by various intracellular pathogens to escape from, arrest or subvert phagosome maturation[9]. Most of these strategies directly or indirectly prevent the final and critical stage of the phagosome maturation process: the fusion of phagosome with late endosomal/lysosomal compartments, which endows them with the bulk of the hydrolytic enzymes and anti-bacterial factors of a mature phagosome[7,8,10]. Analysis of this final and critical step therefore can provide us with strong indicators about the state of maturation of the phagosomes and if the natural physiological state is being positively or negatively affected under the particular experimental settings that are being utilized.

The late endosomal/lysosomal compartments are generally regarded to be the terminal compartments of the endocytic pathway, defined and distinguished from the early endocytic compartments by the presence of various, stage specific, marker molecules. For instance hydrolytic enzymes or membrane components such as Lysosomal associated membrane proteins (LAMPs) among others[11]. The terminal endocytic compartments -henceforth referred to simply as lysosomes- also serve as the main location for the final stages of digestion at the end of the phagocytic/endocytic pathway[12]. In this way, nondigestible probes can be loaded through endocytosis and macropinocytosis from the extracellular milieu and transported through the endocytic pathway to lysosomes, where it accumulates[13,14] after following a defined cycle of uptake and chase. Fluorophore-conjugated probes for fluorescent microscopy such as the organic nondigestible polymer dextran are commonly used as endocyticprobes[15-17].

In this method, we describe the utilization of IgG-coated microbeads as phagocytic cargo to investigate phagosome maturation by analyzing the delivery of lysosomal luminal content to phagosomes. By using a fluorophore-conjugated dextran probe as a readily detectable marker of

lysosomes in live bone marrow derived macrophages (BMM) and time-lapse video imaging with a confocal laser-scanning microscope, we follow the dynamic process of cargo delivery into phagosomes with high temporal resolution. We then describe how the collected time-lapse imaging data can be evaluated using the open-source and freely available image analysis software, Fiji (or ImageJ), statistically analyzed using Microsoft Excel and presented using GraphPad Prism software[14,18].

Following the description of our method, analogous experiments can be designed using modified settings and factors to investigate their influence on phagosome maturation; virtually any other type of adherent primary or cell-line phagocytic cells, genetic loss of function experiments including gene knock-out and knock-down, mutants or expression of fusion proteins, phagocytic-targets, microbead coating compounds, endosomal/lysosomal probes and factors such a cytokines, chemical inhibitors, or siRNA among others are all variables that can be applied to the basic approach of this method.

We define the goal and the basic requirements of this method as follows:

- Goal of the method: to enable direct, quantitative and qualitative observation and analysis of phagosome maturation with high temporal resolution in living phagocytic cells.
- Phagocytic cargo: An appropriately coated microparticle or biological target. Here we use IgG-coated 3 µm spherical polystyrene microparticles that are readily internalized via the Fc-γ receptor mediated phagocytosis. Alternatively, any microorganism or coated microparticle for which a phagocytic receptor is present on the cells can be used.
- Phagocytic cells: Adherent phagocytic cells expressing the appropriate phagocytic receptors. Here we use mouse primary BMMs which abundantly express the Fc-γ receptors. Alternatively, dendritic cells (DC), monocytes or phagocytic epithelial cells or their genetic mutants or knock-out variants could be used.
- Lysosomal cargo: A fluorescently labeled lysosomal probe. Here, Texas Red-conjugated 70,000 kiloDaltons dextran (Dex70kD) is used as the luminal cargo of the lysosomes. Alternatively, any fluorescently detectable marker of a cellular compartment or organelle, or an appropriate probe for phagosomal properties such as acidity or degradative capacity, could be used to study the progression of phagosome maturation.
- Influencing factors: For instance, signaling molecules, chemical compounds, gene knock-down, or transient expression of mutant proteins could. Here, we have forgone the use of such a factor for the sake of simplicity, but for a recent example with mutant protein expression and knock-down influencing factors please see Kasmapour *et al.*[18] Any factor that can affect phagocytosis or the circumstances and mobility of phagosomes and/or the compartments that interact with maturing phagosomes could potentially be used.

Protocol

Animal care and all procedures in this protocol follow the institutional and national guidelines.

Prepare the preparations that are indicated by "П-" beforehand.

1. Preparation of BMMs

1. Perform the culling of the mice by cervical dislocation.
2. Remove femur and tibia bones, free bones from tissue and trim both ends for the accessibility of the bone marrow.
3. Keep bones in ice-cold PBS or ice-cold DMEM medium (supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, on ice until the next step.
   Note: Conduct the following steps under a class II biosafety cabinet to minimize the risk of contamination.
4. Flush the medullary cavity with cold PBS +penicillin/streptomycin using a 26 G (0.45 mm) needle attached to a 1 ml syringe.
5. Pellet cells by gentle centrifugation at 350 x g for 10 min, resuspend the pellet in BMM medium and plate in sterile microbiology noncoated Petri dishes.
   1. Preparation of the BMM medium
      - Dulbecco's Modified Eagles Medium DMEM
      - 10% inactivated FCS
      - 20% mouse fibroblast L929 cell line culture supernatant (source of macrophage colony-stimulating factor, M-CSF)
      - 5% horse serum
      - 2 mM glutamine
   2. Preparation of the L929 cell line culture medium;
      - RPMI (Roswell Park Memorial Institute) 1640 medium
      - 10% inactivated FCS
      - 2 mM glutamine
   3. Preparation of mouse fibroblast L929 cell line culture supernatant;
      - Confluent L929 cells were split 1:4, cultured in 175 cm^2 flasks for 2 days using 20 ml L929 medium and the culture supernatant was collected after 48 hr, filtered and added to the BMM-medium
6. Incubate the cells flushed from the femur in an incubator at 37 °C in 5% CO_2 atmosphere.
7. After every 48 hr (maximum 72 hr) of incubation, aspirate the medium and replace by fresh BMM medium, for up to 10 days. More than 95% of the cells were positive for CD14 as tested by flow cytometry. CD14 is a pattern recognition receptor expressed mainly by macrophages. By determining the percentage of cells positive for this receptor, a pure culture of adherent BMMs has been generated.

Note: prepare and culture the cells accordingly if other cell types are used.

2. Coating of Microbeads as Phagocytic Cargo

1. For preparation of 1 ml bead suspension, use 400 µl of 2.5% 3 µm carboxylated polystyrene microparticles, or alternative microparticles.
2. Transfer the bead suspension to a 1.5 ml microcentrifuge tube, wash 3x with PBS and add 100 µl of 500 mM 2-(N-morpholino)ethanesulfonic acid sodium salt, MES buffer at pH 6.7 to the bead pellet.
3. Dissolve 50 µg mouse IgG (polyclonal IgG antibody) in 50 µl sterile ddH_2O and add to the bead suspension.
4. Fill the suspension up to 1 ml with sterile ddH_2O (450 µl).
5. Incubate the solution for 15 min on a rotating wheel at RT.
6. Prepare EDAC cross-linker, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride in a concentration of 10 mg/ml and add 14 µl to the bead suspension.
   Note: EDAC cross-links amino groups of the IgG with the carboxyl groups on the surface of the beads and facilitates the immobilization of the IgG on the bead surface. This is critical for bead uptake and therefore the solution should be prepared freshly.
7. Incubate the suspension on a rotating wheel for 1 hr at RT.
8. Add 14 µl of EDAC cross-linker and incubate the suspension on a rotating wheel for 1 hr at RT.
9. Centrifuge the suspension at 10,000 x g for 2 min in a microcentrifuge.
10. Wash the beads 3x in stop buffer (1% Triton-X 100 in 10 mM Tris, pH 9.4) in order to stop the cross-linking reaction, by resuspending the pellet in stop buffer and spinning the solution at 10,000 x g for 2 min.
11. Wash beads 3x in PBS and resuspend the pellet in 1 ml PBS.
    Optional: Prepare working aliquots of 30-50 µl.
12. Store the IgG-coated bead suspension at 4 °C for no longer than 4 weeks and never allow the suspension to be frozen.

Note: A preservative such as Sodium azide at a final concentration of 0.02% (w/v) can be added to the suspension to prevent bacterial growth and contamination. In this case washing of the beads prior to its use has to be performed to avoid cytotoxic effects of the preservative. Centrifuge an aliquot of the suspension at 10,000 x g for 2 min and wash the beads by resuspending the pellet in PBS. Repeat washing 3x.

3. Preparation of the Dextran Probe Stock Solution

1. Dissolve the Texas Red 70,000 kiloDaltons Dextran (Dex70kD) in PBS at 20 mg/ml and store at -20 °C, according to the manufacturer's instructions.
   Note: Keep stocks at -20 °C for up to several months, or at 4 °C for few weeks, see the manufacturers documentation.
2. Prepare the dextran solution for the experiment from the stock by diluting it in complete DMEM cell culture medium (DMEM, 10% FCS, 2 mM L-glutamine) at approximately 1 mg/ml (several-fold concentrated solution that will be diluted to the final working concentration of 20 µg/ml before addition to the cells).
3. Use a ultrasound water-bath to sonicate the dextran aliquot for 5 min to homogenize the solution and dissolve aggregates that could later lead to artifact generation during imaging.
4. Centrifuge at maximum g for 5 min in a microcentrifuge to remove any undissolved clumps or contaminants from the solution.
5. Carefully move the supernatant to a fresh tube while avoiding the pellet at the bottom of the tube and dilute the solution to the final working concentration of 20 µg/ml in complete DMEM cell culture medium.
6. Filter-sterile the solution using a 0.2 µm syringe mounted filter.

4. Dextran Preloading

1. Seed cells in a live cell imaging glass bottom dish and incubate for at least 12 hr (at 37 °C in 5% CO_2 atmosphere) to ensure attachment and acclimatization.
   Note: There are segmented glass bottom dishes available that allow performing multiple experiments (up to four compartments) per dish.
2. Prepare the dextran in DMEM solution as described in Protocol 3. Warm up the prepared dextran in DMEM solution, using a water bath at 37 °C.
3. Aspirate medium and add dextran solution to the cells carefully and incubate at 37 °C in 5% CO_2 atmosphere for 2-8 hr for uptake.
   Note: Plan and optimize the protocol based on the probe and cell type that is used and strictly keep to this duration when repeating experiments. This is essential for the profile of dextran accumulation in the endocytic pathway and thus reproducibility of experiments.
4. Wash cells 3x with prewarmed complete DMEM medium; aspirate medium and rinse cells carefully with fresh medium.
5. Incubate cells with complete DMEM medium for 4-12 hr at 37 °C in 5% CO_2 atmosphere.
6. Wash cells once with prewarmed complete filtered phenol red-free DMEM medium.
7. For optimal imaging results, change to prewarmed complete filtered phenol red-free DMEM medium at least 30 min before the start of imaging. Phenol red may cause quenching of fluorescent signals, increasing background noise and thus reduce image contrast and clarity.

Note: Set up the microscope and the imaging settings in advance, according to preplanned and optimized protocol based on the type of probe and cells that are used.

5. Adding IgG-coated Beads

1. Equilibrate an aliquot of preprepared 1% bead suspension to RT and sonicate in an ultrasound water bath for 2-3 min.
2. Add 1-6 µl of the 1% bead suspension to the complete filtered phenol-red free DMEM medium under a class II biosafety cabinet to minimize the risk of contamination (a good starting concentration is 1 µl/2 cm^2 dish surface).
   Note: Here BMMs were seeded at 25,000 cells/well and a 6 µl of 1% stock bead suspension was added to the medium in the dish. This roughly equals a bead/cell ratio of 15:1. The ratio has to be adjusted to the type of the cells that is used and to the bead material; i.e. latex

beads have low density and do not sink to the bottom quickly, whereas polystyrene beads sink and come in contact with the adherent cells much faster and thus in larger numbers.
Note: Depending on the experiment, it may be of advantage to select the region that will be observed in advance. Bead suspension can then be added only to that specific region of the glass bottom dish, therefore increasing the probability of observing favorable uptake events.
3. The dense cloud of beads can be diffused by gently pipetting the suspension in the glass bottom dish.
4. Wait until beads sink to the bottom of the glass bottom dish and are approximately in the same plane with the adherent phagocytic cells.
5. Focus on the region of interest (ROI) and start the time-lapse imaging.

Note: It may be necessary to fine-tune the focus while imaging is in progress, depending on the imaging system that is used, its stability and the mobility of the observed cells. However, note that this may render impossible, the proper analysis of phagosomes that strongly deviate from the plane of focus as a result of refocusing.

6. Imaging

Follow the general guidelines below for optimal imaging quality;

1. Use a confocal imaging system such as a laser-scanning or spinning disc system.
Note: A Leica TCS SP5 AOBS Laser scanning confocal microscope controlled by the Leica LAS AF software and equipped with an environmental control chamber was used here for time-lapse video imaging.
2. Optimize the imaging settings such as scanning speed, magnification, resolution, *etc*. in a manner to allow for a rate of a frame in every 7-20 sec, depending on system used.
Note: Here, a 200 Hz scanning speed, 2x scanner zoom and a line averaging of 2-3x at a resolution of 512 x 512 pixels resulted in a recording time between 3-5 sec/rame. A rate of a frame in every 10 sec was used to record time-lapse movies with durations of at least 120 min.
3. Use appropriate excitation/emission settings based on the used imaging system and probe(s).
    1. Optimize the settings to prevent excessive photo-bleaching.
    Note: Here, the Texas Red Dextran 70kD was excited using a DPSS (561 nm) laser and emission light was detected by a Leica hybrid detector (HyD) at 600-650 nm with the emission peak at 610 nm. Laser intensity has to be adapted to the signal intensity and kept as low as possible to minimize photo-bleaching of the fluorophore(s) and photo toxicity effect of the laser light on the cells. Generally, it is important to balance the scanning speed, line averaging, scanning resolution, frame interval and duration of the imaging to obtain proper and stable signal intensity and capture the complete duration of bead uptake and phagosome maturation process.

4. Include a bright-field (BF) channel for observing the beads if they are not conjugated with a fluorophore.
Note: Beside the Texas Red channel, BF channel was used to visualize the beads, the same DPSS laser was used as the light source and signal was detected with a photo multiplier (PMT) detector.
Note: Make sure to apply identical recording settings when acquiring data that is to be collated. Most important parameters are: excitation laser intensities, detector setting, acquisition settings, magnification and frame intervals. Not using identical frame intervals will necessitate a curve-averaging step as the observation data points will not overlap (e.g. to collate an observation with frames acquired at every 7 sec with another set with frames at every 12 sec). This would increase the steps required for data evaluation and require additional software with curve-averaging function, such as OriginLab's Origin Pro statistics software (http://www.originlab.com/).
5. Preferably, save the time-lapse video in the native file-format of the used imaging system and avoid exporting in compressed formats.

Note: Here, time-lapse movies were saved in the native Leica LAS AF format (.LIF) files, which were then directly imported to open in Fiji. Fiji is able to read the native file formats from most microscope manufacturers using the included Bio formats importer plug-in. Exporting the time-lapse movie file in an image series with JPG or TIFF or as a video file in an AVI container format is not necessary or recommended. In addition to preserving the best possible image quality from the original observation by avoiding lossy compression algorithms (e.g. JPEG), using the native microscope file format insures that the file's meta-data (time stamps, magnification, laser and acquisition intensities, detector settings etc.) is preserved and available to Fiji. However, if for any reason, time-lapse video files need to be exported in a different format for analysis, make sure to use uncompressed file formats such as TIFF image series and separate RGB color (red, green, blue) channels already at this step, as Fiji often cannot successfully separate the BF channel from other color channels in exported files. Also, make sure to preserve the original microscope files as a reference.

7. Analysis of the Time-lapse Movies

1. Use ImageJ, Fiji or any other software that provides an analogues signal association analysis capability.
Note: Here, Fiji was used for the analysis of time-lapse movies. Fiji is an ImageJ distribution containing an image processing package with reorganized tool menus and additional native plug-ins, available for free download at http://fiji.sc. The process described in this section is depicted in **Figure 2**.
2. Open the file in Fiji by clicking "File", "Open" and choosing the file, or by dragging and dropping the file to Fiji.
3. Choose the following options;
    1. Stack viewing: View stack with Hyperstack,
    2. Color options: Color mode Colorized,
    3. Split channels into separate windows: Split channels (for every RGB color-channel that was recorded a separate window will be opened).

4. In case an image series is to be used, load the series to Fiji by going to "File", "Import", "Image Sequence" and choose any image file in the folder that contains the target image series.
    1. Set filters and conditions for loading selected images in the series, for instance;
        1. Number of images: how many frames are to be loaded.

2. Starting image: which image is the starting frame in the series.
3. Increment: the increment between frames to be loaded (every frame, every second frame, *etc*.).
4. File name contains: filtering for specific channel using the file name (for instance by setting "c001", which is how normally the images from "channel 1" are labeled after color-separation of a multichannel time-lapse video).

5. Remove scaling by going to "Analyze", "Set scale…", checking the box "Global" and clicking "Click to remove scale".
   Note: This step allows pixel based analysis of the images in case different magnifications have been used for the various experimental sets that are to be evaluated. If the magnification settings have always been kept constant and the native microscope file was directly imported to Fiji, this step can be skipped, as Fiji can understand and use the magnification and scale data from the microscope file's metadata.
6. Set up measurement parameters by going to "Analyze", "Set Measurements" and checking the following boxes: "Area", "Standard deviation", "Integrated density", "Display label" and "Mean gray value".
7. Redirect to the color channel that contains the signal from the probe that is to be measured and confirm with "OK".
8. Go to the frame in which the measurements are to be started and select the BF channel window by clicking on the upper edge of the window.
9. Use the "oval selection tool" to select a region of interest (ROI), *i.e.* a circular ROI on the internalized bead. Ensure that the selection is fitted tightly to the outer edge of the bead as visible in the BF channel.
   Note: When using a PC, hold the Shift-key while using the selection tool to ensure a circular ROI.
10. Start the measurement optimally a few frames before the full engulfment of the bead is completed and note the frame in which a fully formed nascent bead-phagosome is formed and the phagocytic cup is closed. This should be relatively clearly discernable in the BF channel.
11. Go to "Analyze" and click "Measure" to execute the measurement; the result will be displayed in a separate window titled "Results".
12. Go to the next frame, adjust the position of the ROI in case the bead has moved, and repeat the measurement. The result will be added to the list in the result window; each analyzed frame can be identified based on the value in the "Label" column.
    Note: Using shortcut keys (e.g. "M" for measure) can significantly speed up the evaluation process; see the list of shortcuts and assign costume ones under the menu "Plugins".
13. After completing the measurement of all relevant frames, the results can be copied to a spreadsheet application such as MS Excel. The column "IntDen" depicts the intensities of the selected area in the channel that the measurement was redirected to.

8. Bleaching Correction

Depending on the used probe and imaging settings, photo-bleaching may occur. Depending on its extent, this could strongly affect the outcome of the evaluation. However, this effect can be partially corrected by applying an equal but opposite rate of change to the measured values. If present, the photo-bleaching coefficient can be calculated by measuring the time-dependent signal intensity decrease in the whole frame, or specifically cropped area of the frame, during the time-span relevant to analysis of each phagosome. This process is depicted in **Figures 3** and **4**.

1. Under the "Plugins" menu of Fiji, use the "Time Series Analyzer" plug-in to determine the general decrease in the whole-frame, or ROI specific probe signal intensity over time (**Figure 3**).
   Note: Time Series Analyzer plug-in is available for download at http://rsbweb.nih.gov/ij/plugins/time-series.html, in case not already present in the Plugins menu.
2. Plot the measurement against time (in seconds) in MS Excel, only for the frames that correspond to the frames of an analyzed phagosome.
3. Apply a linear trend-line (**Figure 4A**) to the plot; a negative slope for the decreasing signal indicates the presence of photo-bleaching effect.
4. Apply the reversed slope to values from an analyzed phagosome(**Figures 4B** and **4C**): *Corrected signal intensity (SI) = raw SI + (time in seconds * reverse slope)*

Note: each single analyzed phagosome will have a specific time-span (starting at complete uptake frame) during a recorded time-lapse movie; the photo-bleaching must be recalculated for that specific span and will be only valid for correcting the measured values from that specific phagosome.

9. Data Evaluation

1. Collate the data and calculate the average and statistical error of the bleaching-corrected values in appropriate software.
2. Plot the evaluated data in an appropriate form.

Note: Here, the scientific statistics and plotting software GraphPad Prism (http://www.graphpad.com/scientific-software/prism/) was used. Prism software is able to generate and plot an average curve with the corresponding error indicators as long as all the data have the same frame rate (i.e. all were acquired with at the same intervals, such as one frame every 10 sec).

Note: Wide variations of the absolute SI values within repeats of the same experiment set will introduce very large statistical error and render the data unusable. This can be the case if the protocol, *e.g.* imaging settings, are not held strictly identical among different experiment repeats that are to be collated.

Representative Results

The correct preparation of the cells and the beads for imaging is critical. **Figure 1** shows the outline of the seeding, probe-loading and initial imaging steps in this method, based on our optimized protocol for BMMs. It is essential therefore that the protocol parameters be tested and optimized depending on the type of cells and probes that are to be used.

After addition of the IgG-coated beads to the preloaded cells, it is important that the field of view is chosen in a manner to provide the largest number of potential uptake events in the largest number of cells possible, while keeping to the predefined magnification setting of the protocol.

As depicted in **Figure 2**, this approach can provide for a larger number of analyzable phagosomes in each time-lapse video. In addition to increasing the data points per video yield of each experiment set, this decreases the variation introduced by varying peripheral conditions and increases the robustness of the data.

It is recommended that the signal-association measurement procedure (**Figure 2**) be carried out by the same person and in a blind manner if possible. This could help to reduce the error by eliminating multiple personal-error sources and reduce the bias, as the ROI assigned to each phagosome is to be selected and moved manually after each frame.

While naturally the rule of "the larger the sample size, the better" also applies here, we avoid evaluating more than 5 bead-phagosome per cell in the field of view to prevent giving too much weight to a single cell as the behavior of all cells within the same culture is not necessarily homogenous[19]. These phagosomes should be selected randomly as far as possible. In this context, parameters such as phagosomes being fully visible in the focal plane for the whole duration of imaging and also not being affected by excessive photo bleaching are essential. Moreover, uptaking a large number of large particles by a single cell can affect the fusion dynamics for the phagosomes in that cell, so the priority should be given to the phagosomes that are uptaken earlier in the process by a relatively "empty cell". As a general rule, the averaged measurements from at least five cells from up to five independent experiments (hence, up to 25 phagosomes) should provide for good statistical significance.

The application of Time Series Analysis or a comparable method to detect a possible photo-bleaching effect (**Figure 3**) is very important, as some probes will suffer from strong bleaching while some are very photo-stable. The photo-bleaching correction step (**Figure 4**) should only be applied if a strong bleaching effect is observed in all experiments with the same probe. It has to be noted that this process can only partially correct the effect of bleaching. Importantly, excessive bleaching in only a few cases can be a sign of nonoptimal conditions, such as wrong excitation or imaging settings, wrong medium pH, unhealthy cells, or nonfresh reagents.

In **Figure 5C**, the bead-phagosome indicated with an arrow in the **Figure 5A** and in **Figure 5B**, is analyzed and the Dex70kD signal intensity (SI) association to maturing phagosome during a span of 90 min is plotted. The source of noise in the curve is the frame to frame variations of the measured signal due to factors such as obstruction of the analyzed phagosome by other phagosome and focal plane fluctuations. However, the temporal trend of signal association to the phagosome is readily clear. After averaging the analysis from 10 phagosomes from the two cells visible in **Figure 5A**, an average curve can be plotted (**Figure 5D**) for which the statistical error can be plotted as the standard error of mean (SEM) or the standard deviation (SD) depending on the sample size and experimental conditions. While the absolute SI values of the averaged curve is usually different from that of any single analyzed phagosome, the temporal trend is normally very similar. After analysis, it can be concluded that the delivery of Dex70kD as lysosomal luminal cargo to bead-phagosomes in wild type BMMs reached a plateau within the 35-40 min after phagocytosis.

This method can therefore be employed to investigate the qualitative or quantitative effect of various factors on phagosome maturation process.

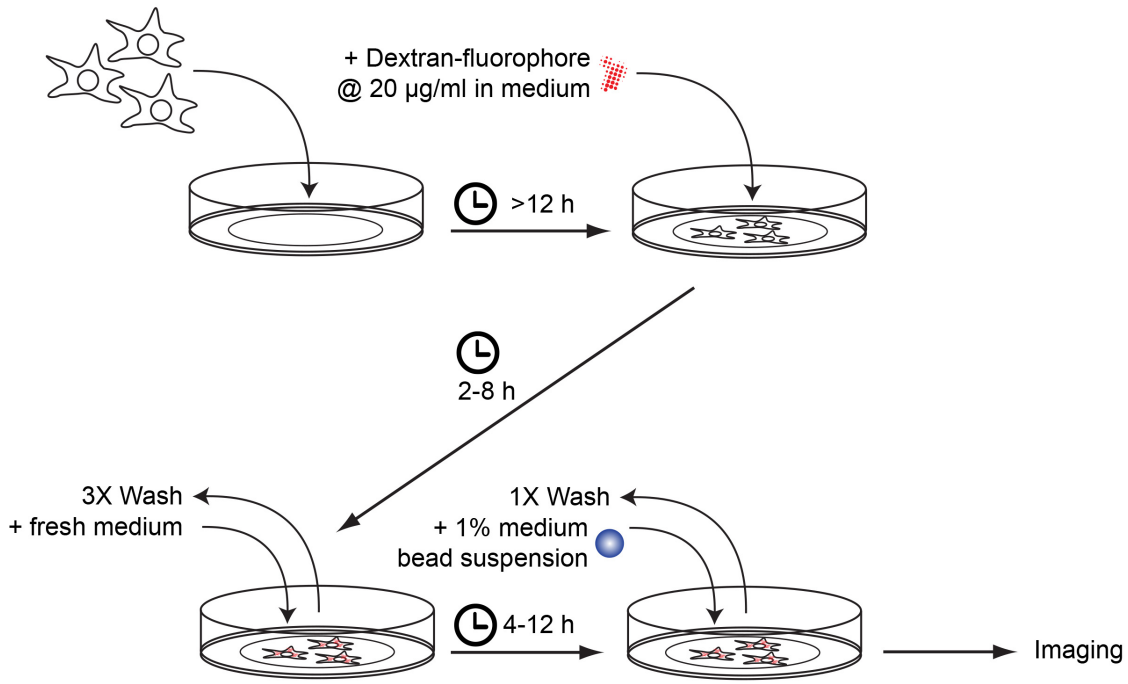

Figure 1. Preparation of cells for imaging. Preprepared BMMs are seeded in a glass bottom dish at least 12 hr before addition of dextran, solution of dextran in medium is added at 20 µg/ml concentration and incubated for 2-8 hr (loading), cells are washed, fresh medium is added and cell are incubated for at least 4 hr (chase), cells are washed again and bead suspension is added just prior to the start of imaging. Click here to view larger image.

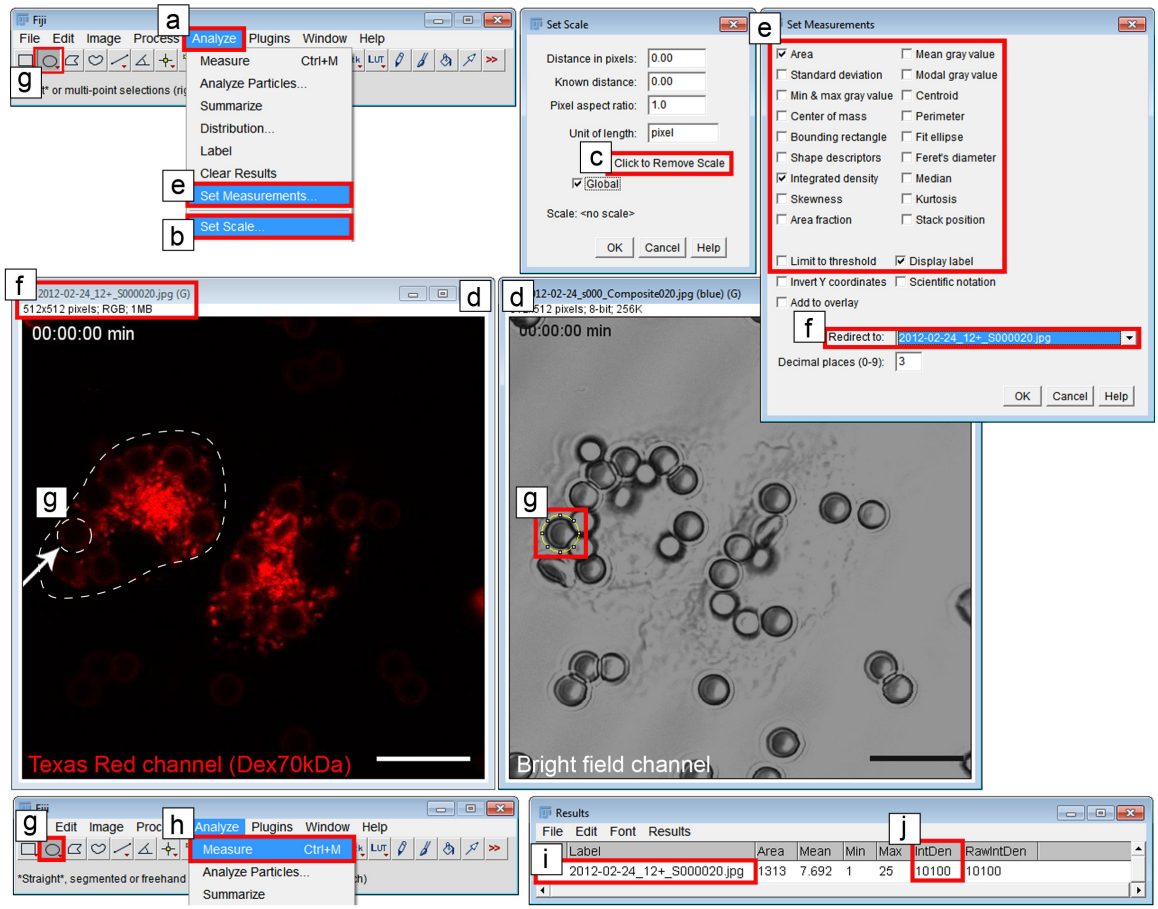

Figure 2. Step by step instruction for image analysis. After loading the time-lapse video or image sequence in different channels in Fiji, the evaluation follows in these steps (Note that the figure depicts an image collage and not all the windows shown here will remain open simultaneously). (**a**) Under "Analyze" menu, pixel to distance scaling is reset in case the meta-data of the images are not available to Fiji, different magnifications has been previously used in Fiji or the time-lapse recordings are at different magnifications, by going to (**b**) "Set Scale", (**c**) ticking the "Global" box which applies the reset to all subsequently loaded image series and clicking on "Click to Remove Scale". (**d**) Make sure that both the bright field (BF) channel image series (where bead-phagosomes are clearly visible) and the channel containing probe signal are loaded and have the exact same frame-counts and pixel dimensions, *e.g.* two series of each 400 frames with 450 x 450 pixel dimensions. (**e**) Under "Analyze" menu, parameters of evaluation can be set under "Set Measurements", where "Area", "Integrated density" and "Display label" parameters have to be selected. (**f**) In the same window, while having both relevant channels image-series open, under "Redirect to" field, choose the name of the probe-signal channel image series (Texas Red channel here). (**g**) This ensures the measurement of the red-channel intensity in the same Region of Interest (ROI) that is indicated by the dotted line and the white arrow in red-channel. The Circular ROI is selected in the BF channel using the "Oval" selection tool. (**h**) Start the measurement under "Analyze", "Measure" or using the shortcut command, and repeat for every frame of the whole series for the desired duration of experiment (*e.g.* 90 min). In each frame, move and readjust the ROI location to the bead-phagosome of the interest and note that going to next frame in the BF series and giving the "Measure" command automatically measures the ROI in the corresponding frame of the red-channel. (**i**) Measurement results will appear as a list in the "Results" window. Under the "Label" column, the exact frame for each line is clearly identified; use this to check for repeated measurement of a single frame or skipped frames in long image series. (**j**) The "IntDen" short for integrated density is the critical output parameter; copy at least the label and the "IntDen" values to a MS Excel sheet to continue with the evaluation. The unit of IntDen value is undefined and indicated as arbitrary units (a.u.), this does not create any problems as long as the protocols are strictly followed. Scale bar is 10 µm. Click here to view larger image.

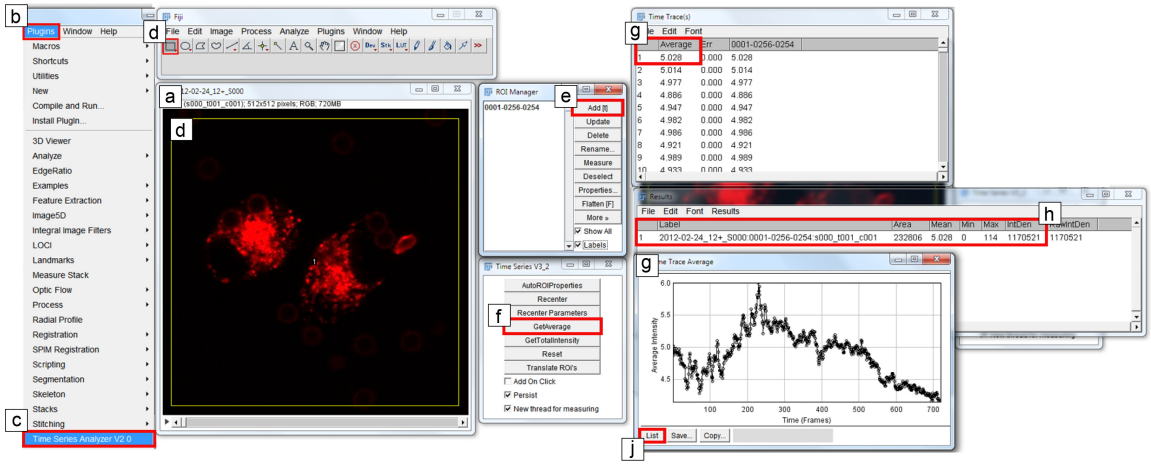

Figure 3. Analysis of photo-bleaching. (**a**) Open image series in the channel of interest and make sure the scale has been removed if needed, (**b**) Under "Plugins" menu click(c) "Time Series Analyzer". (**d**) Using the rectangular selection tool, draw a rectangular ROI covering almost the whole frame, or at least the entire cell of interest for the entire duration of the movie, (**e**) add the selected ROI, (**f**) click "Get Average" and (**g**) Results will be displayed in the "Time Traces" table and "Time Traces Average" plot. Note however, that only an "average" (Mean intensity) value is plotted against the frame number and not the "IntDen" value. (**h**) Under "Analyze" menu, run "Measure" without changing any other parameters, to measure the "IntDen" for the exact same ROI at the same frame. This will provide the equivalent of the Mean intensity in "IntDen", use this value to calculate the conversion factor (equal to the "Area") and plot the "IntDen" of the whole-frame ROI against time in seconds in MS Excel. (**j**) Click "List", copy the list of values to an Excel sheet and convert all "Mean" values to "IntDen" values. Click here to view larger image.

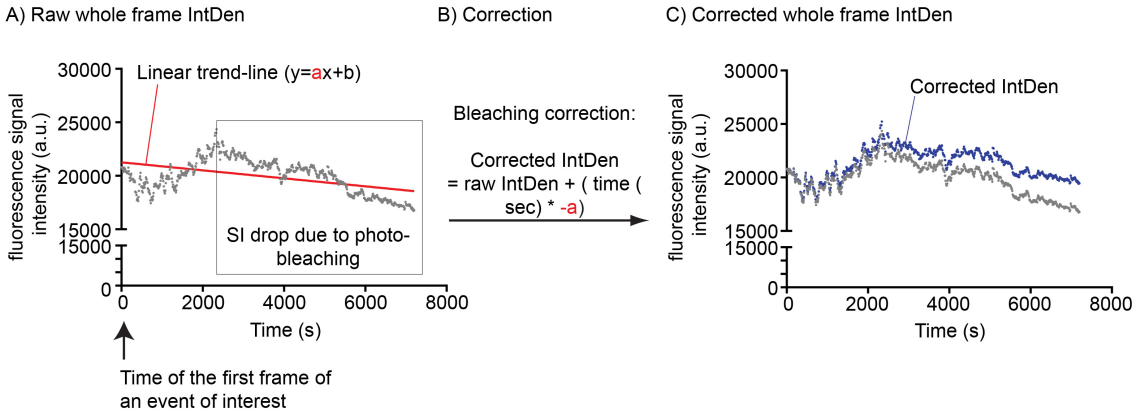

Figure 4. Correction of photo-bleaching. Depending on the used probe and imaging settings, photo-bleaching may occur. This could strongly affect the outcome of the evaluation. **A**) Raw IntDen values (in a.u.) of the probe from the whole frame are plotted against time in seconds in MS Excel. Add a linear trend-line; a clearly negative slope indicates the presence of photo-bleaching effect. **B**) As an example, applying the reversed slop on the raw IntDen using the correction formula yields the corrected IntDen. **C**) Corrected IntDen values are partially compensated for the effect of photo-bleaching. Click here to view larger image.

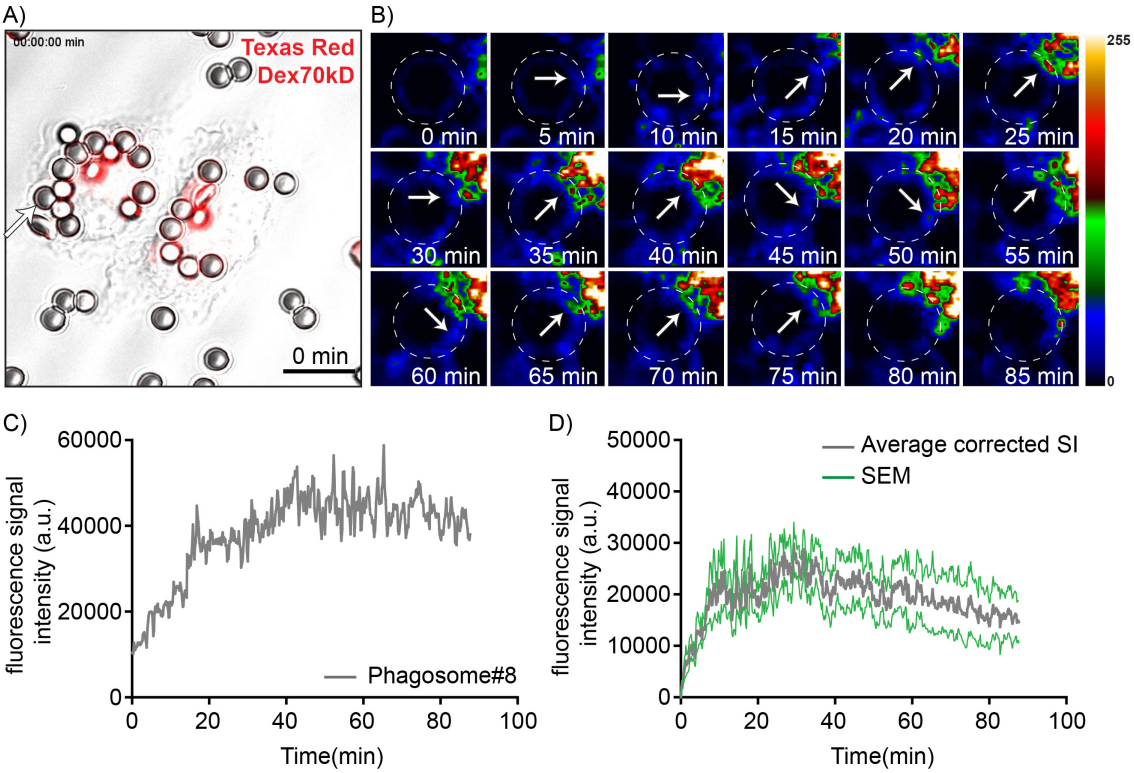

Figure 5. Presentation of representative cells, kinetics and averaging of the corrected signal. A) BMMs loaded with dextran probe at min 0 upon uptake of the IgG-coated bead indicated by the arrow. Scale bar is 10 µm. Corresponds to **Movie 1**. **B**) 2.5X zoomed insert from a time-lapse movie, depicting the indicated phagosome over a span of 85 min after uptake, false-colored for better visibility with "thal" Look-up-table (LUT) in ImageJ. The measured phagosome ROI is indicated with the white dashed line. The instances of dextran delivery and accumulation in the phagosome are indicated with white arrows. **C**) Corrected fluorescence signal of Texas Red 70kDa dextran delivered from lysosomes to the indicated phagosome. **D**) Averaged fluorescence IntDen values from 10 phagosomes within the two indicated cells ± SEM. Click here to view larger image.

Discussion

In the following section we will discuss critical steps of the presented method and its limitations. Further, we will connect some of the common problems with their solutions, introduce possible modifications and consider advantages of our method as well as suitable complementary methods for this approach.

The preparation of the beads, including the coupling of IgG to the bead surface, is crucial and the use of unfresh preparations should be avoided. In additions to that, it is also critical that the dextran is prepared from the frozen stock (diluted and sterilized) freshly before every experiment. It is important that the loading of the dextran follows a precise schedule to achieve consistency in cellular uptake and compartment distribution and accumulation. In addition, it is important to use not only the same microscopy setup and software settings, but also to keep the peripheral conditions as constant as possible (*e.g.* temperature, CO_2, humidity, bead-addition technique, *etc.*). Another critical point is the selection of a field of view or the cell of interest, the sample should be scouted before start of imaging in order to choose cells that are representative.

One of the major limitations of the method is the number of experiments necessary to yield significant samples sizes. The described method is relatively time and work intensive, since the number of observable phagosomes in one field of view, depending on the chosen resolution, is limited. Pooling the results of a very low number of randomly chosen phagosomes entails the risk that a few extreme outliers can have a prominent effect on averaged values. Larger sample sizes on the other hand will enable masking of phagosome-to-phagosome or cell-to-cell variations.

The common problems that may occur include contaminations, lack of phagocytosis and excessive bleaching and photo toxicity effect due to the laser light.

Sterility, proper storage and preparation of the dextran are critical to avoid contaminations. In our experience, the purchased dextran should not be considered as a sterile solution. The risk of sample contamination can be significantly reduced by extensive centrifugation of the stock and sterile filtration of dilute dextran solution. On the other hand, regular preparation of fresh bead suspension, which contains proteins and is susceptible to contaminations, can also reduce the risk of contaminations.

Lack of or low levels of phagocytosis could be due to suboptimal IgG-coating of the beads. Therefore, it is important to prepare the components for the IgG coupling of the bead (IgG solution, EDAC) freshly and not to use beads which are more than 4 weeks old or not have been kept exclusively at 4 °C. Since the beads are briefly sonicated before utilization, it is recommended to prepare working aliquots to avoid repeated sonication of the stock suspension. Another reason for lower than expected phagocytic performance could be unhealthy, suboptimally cultured or

old cells; strict adherence to recommended culture conditions for the used cell type is therefore essential. For instance, excessive trypsinization during harvest could damage the phagocytic surface receptors and impair phagocytic capacity.

Excessive photo-bleaching may be a major problem with some fluorophores. Adjusting microscope settings, *i.e.* the intensity of the excitation light and duration of sample exposure, according to the bleaching susceptibility of the fluorophore helps to control the bleaching. With regard to the time interval between acquisitions of successive frames, a balance between the frame-rate, which at a higher frequency provides for higher temporal resolution and better revelation of important interaction dynamics, and the bleaching of the probe has to be stricken. The optimal balance primarily depends on the questions that are to be addressed in the given experiment. Adjusting the scanning resolution, scanning frequency and line or frame averaging represent settings that can be changed to further optimize the duration of sample exposure to the excitation light. Availability of some of these options is however determined by the type of microscope system that is being used.

The same general guidelines should be followed to minimize the photo-toxic effect of laser excitation light on the cells. This effect can be manifested as death of the cells exposed to the laser light during the imaging or a marked increase in unnatural morphologies[20].

Here we have presented a basic and general method, which allows for modifications and adaptation to different experimental settings. As mentioned, this method can be employed for loss-of-function studies such as in knockout models, gene knockdown or mutant protein expression. It also allows for different opsonization strategies to target specific phagocytic receptors, the use of different endosomal/lysosomal probes as well as the study of chemical inhibitors or cytokines.

Over expression studies with various fluorescent fusion proteins and their mutants can be used to investigate effects on delivery of an appropriate probe to the phagosomes. Furthermore kinetics and dynamics of interaction of the protein itself with phagosomes can be analyzed. There are several probes available that can be utilized for these purposes. A prominent example of common class of probes include the LysoTracker group of acidotropic dyes, which are widely used to track protonation lumen of endosomal compartments as well as the phagosomal lumen. The coating of the beads offers another multitude of possibilities. Ligands, including nucleic acids such as specific CpG sites of bacterial DNA[21], bacterial surface components such as LPS, proteins such as avidin, serum proteins including complement factors (*e.g.* C1q or iC3b) all can be cross linked to beads[22-24]. This allows the study of the role of these ligands in phagocytosis and phagosome maturation. Using signaling molecules and immune mediators (for instance cytokines) open another range of possibilities.

Finally, this method can also be expanded and adapted to conduct direct studies with microorganisms, for instance by comparing the uptake of live vs. killed or pathogenic vs. nonpathogenic bacteria, although the irregular shape of bacteria poses new challenges during image analysis.

Future applications of this method are as manifold as the modifications that can be applied. As a next step, ratio-metric analysis methods can be adapted. Combination of pH sensitive and pH-insensitive florescent dyes for bead-coating, or making use of single probes to measure phagosomal pH (*e.g.* pHrodo, FluoProbes) as well as compounds that make it possible to experimentally follow the kinetics of protein and lipid degradation inside the phagosome (*e.g.* OVA, HRP, bovine serum albumin and triglyceride lipase substrates) are available[25-31]. These together make possible the establishment of a vast number of investigative approaches based on the basic method of live cell imaging of phagosomes presented here.

The described method can achieve far higher temporal resolutions compared to approaches that are based on phagosome isolation[32]. ELISA or Western Blot analyses of phagosomes can represent very high numbers of events at particular time points, but the data represent a far more heterogeneous and potentially unsynchronized population of events. Flow cytometry based approaches theoretically allow the analysis down to the individual cell level and in that way may be less prone to heterogeneity of cell populations but the similar problem of uncertain synchronicity and the need for very high event counts for reliable evaluation still persist. Nonetheless, the high throughput, sensitivity and reproducibility of flow cytometry approaches[33,34] make them an optimal complement to the live cell imaging approach.

Taken together, the advantage of our method lies in accuracy and high temporal resolution with which single phagosomes can be followed in their maturation process in living cells. Compared to all other approaches which restrict observations to fewer time points, in nonviable and pretreated cells, it is possible here to follow cellular events dynamically and continuously over long periods of time under readily modifiable physiological conditions. One could define events relative to the uptake of the phagocytic cargo, which allows discrimination of the stages of phagocytosis more precisely. In that way, synchronization enables to not only visualize similar behavior of phagosomes; it also allows identifying individual differences due to *e.g.* cell-to-cell variations. Most importantly, dynamics of interactions which may hold the key to understanding complex interactions can be effectively illuminated in high temporal resolution using our live-cell imaging approach.

Disclosures

The authors have nothing to disclose.

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