Chapter 7

Intracellular Manipulation of Phagosomal Transport and Maturation Using Magnetic Tweezers

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Abstract

Phagocytosis is an important process of the immune system by which pathogens are internalized and eliminated by phagocytic cells. Upon internalization, the phagosome matures and acidifies while being transported in a centripetal fashion. In this chapter, we describe protocols for simultaneous imaging of phagosomal acidification as well as their spatial manipulation by magnetic tweezers. First, we describe the protocols for functionalization of magnetic microbeads with pH-sensitive dyes and pH calibration of these particles. We also describe the preparation of magnetic tweezers and the calibration of forces that can be generated by these tweezers. We provide details of the design of the custom electrical and optical setup used for simultaneous imaging of phagosomal pH and phagosome's location. Finally, we provide a detailed description of the data analysis methodology.

Key words Phagocytosis, Magnetic tweezers, Intracellular manipulation, Acidification, pH-sensitive fluorescent dyes, Quantitative biology

1 Introduction

Phagocytosis is a process by which immune cells like neutrophils and macrophages capture and eliminate pathogens [1, 2]. Via this process, phagocytic cells internalize pathogens in an organelle called the phagosome. The pathogen-containing phagosome then undergoes phagosomal maturation. During the maturation process, both the phagosome's membrane and its lumen evolve as a result of intricately controlled membrane fusion and fission events with lysosomes. Phagosome maturation is marked by a gradual acidification of the phagosomal lumen as a result of the V-ATPase proton pumps in the phagosomal membrane. During a few minutes, the phagosomal pH drops from near neutral to a pH of about 4.5–5.0 over the duration of a few minutes [1].

During the maturation process, the phagosome gets attached to the cytoskeletal network and is transported in a centripetal fashion from the cell periphery to the perinuclear region of the cell [3, 4].

Roberto Botelho (ed.), *Phagocytosis and Phagosomes: Methods and Protocols*, Methods in Molecular Biology, vol. 1519,
DOI 10.1007/978-1-4939-6581-6_7,

Although phagosomes have been demonstrated to exhibit bidirectional motion consisting of both dynein-mediated centripetal motion (towards the minus end of microtubules) and kinesin-mediated centrifugal motion (towards the plus-end of microtubules), the centripetal motion however appears to be preferred [5]. While the centripetal motion and localization is a well-established phenomenon, the reason behind this transport and its relationship to phagosomal maturation remains poorly understood. Centripetal transport has mainly been linked to the high concentration of lysosomes in the perinuclear region. A number of methods have been utilized to visualize lysosome localization including their direct visualization using GFP-tagged Lamp1 and Lamp2 proteins [6]. Lysosomes have also been labeled using acidotropic fluorescent probes like LysoSensor [7] and LysoTracker [8]. All these techniques have demonstrated that although the lysosomes are distributed throughout the cell, they are present in substantially higher numbers in the nuclear periphery. This has led to the expectation that phagosomes travel to the perinuclear region to promote phagosome-lysosome fusion [6], which in turn leads phagosomal maturation and eventual pathogen elimination.

To examine this reasoning, a number of approaches have been used for manipulating phagosomal location to study the relationship between transport and maturation. Most studies either involve disrupting the cytoskeleton–actin and microtubular tracks on which the phagosomes are transported—using drugs like cytochalasin and nocodazole or by disrupting various proteins that are part of the machinery involved in the transport process e.g. molecular motors [9, 10]. Although these drug-based methods inhibit centripetal transport, biochemical intervention also affects a myriad of other cellular processes that require an intact cytoskeleton. For example while microtubular disruption by nocodazole prevents phagosomal transport, it also disrupts all long-range intracellular transport and affects basic cellular processes like cell-division. It is therefore imperative to design an experiment that allows manipulation of phagosome location with minimal side effects on other cellular processes.

We addressed this problem using a combination of magnetic tweezers and magnetic microbead-containing phagosomes [11]. While phagosomal maturation is followed by measuring the pH of the acidifying phagosomal compartment using pH-sensing magnetic microbeads, magnetic tweezers are used for spatially manipulating microbead-containing phagosomes.

Phagosomal pH has been traditionally measured using ratiometric approaches. This approach requires a combination of two fluorescent dyes, one of which is pH-sensitive while the other is pH-insensitive. The ratio of intensities of these two fluorophores is a measure of pH. While Oregon Green and Fluorescein are the two commonly used pH-sensitive dyes, Texas Red and

Carboxyltetramethyl Rhodamine are the common pH-insensitive dyes used in ratiometric studies [12]. In this study however, SNARF-4F fluorescent dye has been used. The emission spectrum of this dye consists of two pH-dependent peaks which exhibit pH-dependent changes in intensity. The ratio of these two intensity peaks is a measure of pH. This eliminates the requirement of a second pH-insensitive dye. In this study, we delivered the pH-sensing dye to the phagosomes by attaching the dye on microbeads and allowing the functionalized microbeads to be phagocytosed [12]. A wide variety of microbeads can be used for studying phagocytosis. The majority of studies so far have used polystyrene/latex microbeads [13]. The main advantage of these microbeads is that they are by themselves chemically inert and they can easily be functionalized with surface-reactive chemical groups which can be used for immobilizing other ligands through cross-linking reactions. A pH-sensing fluorophore can thus be attached to the microbeads. The microbeads' size and surface chemical reactivity can also be easily varied depending on the phagocytic assay of interest. In case of quantitative imaging assays, the microbead autofluorescence should also be considered (*see* **Note 1**).

Additionally, given the requirement for magnetic manipulation, microbeads also had to be magnetic in nature. Instead of latex microbeads, superparamagnetic microbeads were therefore used as they get magnetized (i.e. exhibit magnetic moment) only in the presence of an external magnetic field. Once magnetized, they experience a magnetic force when placed in an external field gradient and can therefore be physically manipulated (*see* **Note 2**). In addition to spatial manipulation, the translational and rotational movement of the magnetic particle in a magnetic field can also be used to study micromechanical properties of phagocytosis [11, 14].

In order to spatially manipulate magnetic microparticles in the intracellular milieu, a single-pole magnetic tweezers setup was developed. Magnetic tweezers have an important advantage over other approaches: the magnetic tweezers are very selective to magnetic materials only and as a result the force is selectively applied only on the organelle containing the magnetic microbead (i.e. phagosomes) [15]. Magnetic tweezers are based on the principle that a magnetic microbead placed in a magnetic field gradient experiences a translational force in the direction of increasing magnetic flux. It is possible to exert forces on the order of several nN on micron-sized magnetic microbeads placed in magnetic field gradients [16]. A magnetic microbead of moment $\vec{m}$ placed in an external magnetic field of flux density $\vec{B}$ experiences a force. $\vec{F}_{\text{mag}} = \nabla\left(\vec{m} \cdot \vec{B}\right)$ The amplitude of the magnetic moment $\vec{m}$ of a microbead scales with its volume ($|\vec{m}| \propto d^3$, with d being the microbead diameter). To be able to apply high enough forces on micron-sized small microbeads, it is therefore essential to generate magnetic fields with large enough gradients [17].

For generating a magnetic flux gradient, the magnetic tweezers setup consisted of three functional parts: generation of the magnetic flux, transporting the flux to the area of interest and producing the required gradient in the flux density [18]. Although magnetic flux can be generated using permanent magnets, this approach suffers from the limitation that it can neither be changed in amplitude nor be rapidly turned on and off. The only way to change or switch off the magnetic flux of a permanent magnet is to physically (re)move the magnet, which can be rather complicated and can also lead to disturbances in the experiment. For this reason, magnetic flux is generated electromagnetically by passing a current through a copper coil. Since the magnetic flux is controlled by the electric current passing through the coil, not only can the magnitude of the generated flux be varied but it can also be rapidly switched on or off by turning the current on or off. The generated magnetic flux is then transported via an iron rod with a micromachined sharp tip. The generated flux depends, among others, on the geometry of the tip.

Below, we describe the detailed protocol for preparation and calibration of pH-sensitive magnetic microbeads as well as a single-pole magnetic tweezers setup [11, 12]. We also provide details of the design of the electrical and optical setup used as well as a description of the data acquisition and analysis methodology.

2 Materials

2.1 Cell Culture

1. The experiments described here were performed with murine macrophage cell line RAW 264.7 (ATCC catalogue number: TIB-71). These cell lines were transfected with Kmyr-GFP.
2. DMEM culture medium without Phenol Red and supplemented with 10% Fetal Bovine Serum, 2 mM L-Glutamine and Pen-Strep (*see* **Note 3**).
3. Standard tissue culture flasks.
4. Chambers (petri dish) with a cover glass bottom (Cellview, Greiner Bio-One) for microscopy experiments.
5. Cell scrapers.

2.2 Bead Functionalization

1. Dynal M270 microbeads with amine surface functionalization (Thermo Fisher, USA). Store at 4 °C.
2. SNARF-4F 5-(and-6)-Carboxylic Acid Fluorescent dye (Thermo Fisher, USA). Dissolve in DMSO to 2mM concentration (final volume 1ml). Divide in 20 equal parts in microcentrifuge tubes. Let DMSO evaporate in a desiccator, store tubes at −20 °C (*see* **Note 4**).

3. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4 with 1 M NaOH. Store at 4 °C for up to 6 months.
4. 2-(N-morpholino) ethanesulfonic (MES) buffer: 50 mM MES in water with pH adjusted to 5.7 (Sigma Aldrich, USA). Store at 4 °C for up to 6 months once freshly prepared.
5. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma Aldrich, USA). Store at −20 °C.
6. *N*-Hydroxysuccinimide (NHS) (Sigma Aldrich, USA). Store at −20 °C.
7. 30 mg/ml Human IgG (Jackson Labs, USA). Store at 4 °C for up to 6 months.
8. pH Buffers for pH calibration. These are commercially available. Alternatively, citric acid ($C_6H_8O_7$) and disodium hydrogen phosphate (Na_2HPO_4) can be mixed to prepare pH-buffers in the range pH 3–pH 8 (*see* **Note 5**). Store at 4 °C for up to 6 months.

2.3 Magnetic Tweezers

1. Soft Iron sheets for magnetic pole preparation. These should be available at any metal supplier.
2. Copper coil (5 mm × 5 mm, rectangular cross-section) with 250 turns to be wrapped around a 5 mm × 5 mm iron core.
3. Three-axis Oil Hydraulic Micromanipulator (Narishige, Japan).
4. Home-built amplifier with four PA39 power amplifiers (Apex Microtechnology Corp.).
5. Glycerol for force calibration.
6. A computer for controlling the amplifier.

2.4 Optical Setup

1. A widefield fluorescence microscope.
2. LED for fluorophore excitation (Lumileds, 520–550 nm).
3. Near infra-red (NIR) long-pass filter (>750 nm) (Chroma Technology Corp.).
4. Band-pass filters (570–615 nm, 655–740 nm) (Semrock).
5. Dichroics and mirrors (Semrock).
6. Filter-wheel with 6-positions (Thorlabs).
7. Video camera (WOTEC 902H).
8. An intensified CCD camera (Princeton Instruments Roper Scientific PentaMAX 512 FT).
9. Temperature-controlled microscope incubator (Solent Scientific, UK).

2.5 Software

1. ImageJ (National Institute of Health) for image processing.
2. MATLAB (MathWorks) for data processing.
3. LabVIEW (National Instruments) for hardware control as well as data processing.

3 Methods

3.1 pH-Sensitive Magnetic Microbeads

3.1.1 Bead Functionalization and Opsonization

Dynal M270 magnetic microbeads (*see* **Note 2**) were functionalized with SNARF-4F fluorescent dye and opsonized with IgG as follows:

1. Resuspend 20 μl of stock (30 mg/ml) Dynal M270 microbeads solution in 250 μl of MES buffer at pH 5.7.
2. Wash twice with 250 μl MES buffer to ensure removal of traces of storage buffer that the beads were supplied in. To do this, add 250 μl MES buffer to the beads and hold a permanent magnet close to the bottom of the microcentrifuge tube. Once the beads get collected at the site close to the magnet, the supernatant can be removed. Resuspend the beads in fresh buffer.
3. Remove the buffer and resuspend the beads in MES buffer supplemented with 50 mM *N*-Hydroxysuccinimide (NHS) (*see* **Note 6**).
4. Mix SNARF-4F dye in MES buffer to a final volume of 25 μl and final dye concentration of 4 mM in a separate microcentrifuge tube.
5. Combine the solutions from **steps 3** and **4** and mix well. Since the carboxylic group on the fluorophore is inert, no reaction occurs at this stage.
6. Add 20 mg EDC to the above bead solution to activate the carboxylic groups on the fluorophores and enable the formation of the amide bond between the fluorophores and the beads. Mix well by pipetting and then incubate for 2 h at room temperature in the dark, with gentle mixing on a rotator.
7. Wash microbeads five times using magnetic separation to remove excess dye and resuspend them in PBS buffer at pH 7.4.
8. Opsonize the dye-functionalized microbeads via nonspecific binding of human IgG by resuspending microbeads in 240 μl of PBS and adding 10 μl of antibody (1.2 mg/ml final concentration).
9. Incubate further for 1 h in the cold room (4 °C) with gentle mixing.

10. Wash microbeads three times with PBS to remove free IgG and then resuspend in 250 μl PBS. These beads can be kept at 4 °C for up to 1 week (*see* **Note 7**). Microbeads thus prepared are referred to as "M270-SNARF" microbeads in the rest of the text.

3.1.2 M270-SNARF Beads pH Calibration

The emission spectrum of SNARF-4F dye consists of two pH-dependent peaks (570–615 and 655–740 nm) which exhibit pH-dependent changes in intensity. The ratio of these two intensity peaks is used as a measure of pH. Prior to using these microbeads for measuring phagosomal pH, their pH-dependence was calibrated against buffers of known pH (*see* Fig. 1) as follows:

1. Prepare pH-buffers in the range pH 3–pH 8 (*see* **Note 5**).
2. Add 1 ml of pH 3 buffer in a petri dish with a cover glass bottom. To this, add 1 μl of SNARF-M270 beads. Allow the beads to settle at the bottom of the chamber on the coverslip.
3. Use any widefield fluorescence microscope to illuminate the beads with a light source at ~543 nm. Acquire an image each in the two pH-dependent peaks (Channel 1: 570–615 nm and Channel 2: 655–740 nm) using appropriate band-pass filters (*see* Fig. 7b, c).
4. A custom-written macro script in ImageJ can be used for further image analysis. Select one microbead and measure its integrated intensity over its entire surface in the two channels (see Image Analysis section for detailed methodology).

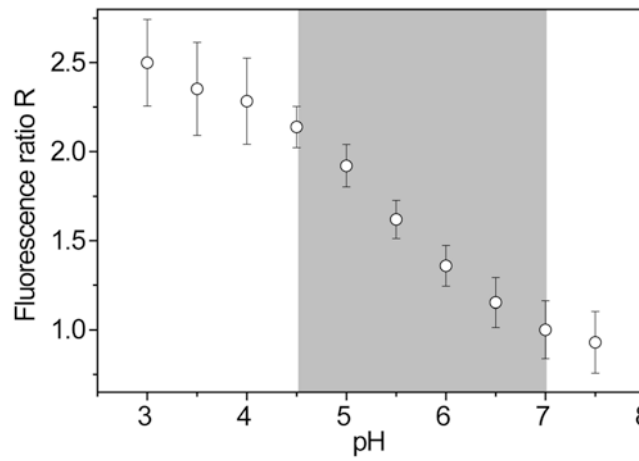

Fig. 1 Calibration of pH-sensitive magnetic beads against buffers of known pH. Mean fluorescence ratio *R* of SNARF-M270 microbeads in different pH-buffers (error bars are standard deviations). The grayed area indicates the relevant physiological pH range for phagocytosis (pH 4.5–7.0). This calibration curve can be used for ratiometric imaging of phagosomal pH. The fluorescence ratios *R* are normalized such that ratio $R = 1$ at pH 7. Adapted from Shekhar et al. [12] with permission from Elsevier

5. Determine the fluorescence ratio R for the microbead by dividing the integrated intensity of the microbead in Channel 1 by the integrated intensity of the microbead in Channel 2.
6. Repeat **steps 4** and **5** for at least 50 more beads over a few fields of view.
7. Repeat **steps 2–6** for buffers with varying pH up to pH 8. Calculate the mean ratio R for each pH value.
8. Draw a calibration curve of fluorescence ratio R as a function pH (*see* Fig. 1). This calibration curve can be used to convert ratio R in to phagosomal pH.

3.2 Magnetic Tweezers: Design and Development

The magnetic tweezers used here consists of following physical parts: a magnetic pole, an electric coil to magnetize the pole and an amplifier or current generator.

3.2.1 Magnetic Tweezers Pole

A single pole magnetic tweezers consisting of an iron rod with a sharp tip was used. It was prepared as follows:

1. Cut rectangular rods with dimensions of 5 mm × 5 mm × 150 mm from commercially available soft iron plates.
2. Micro-machine one end of the rod with a lathe in the workshop to an approximate parabolic shape with a feature size of about 100 μm (*see* Fig. 2).
3. Prepare multiple tips of varying curvature in order to generate a range of magnetic field gradients (*see* **Notes 8** and **9**).
4. To allow very precise movement of the tweezers tip across the microscope field of view, attach the magnetic tweezers to a micromanipulator.

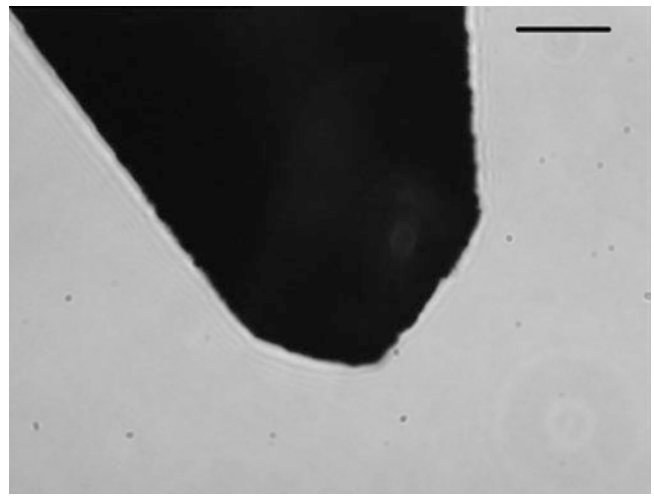

Fig. 2 Magnetic tweezers pole tip. Microscopic image of the micro-machined iron tip of the magnetic tweezers pole. Scale bar: 50 μm

3.2.2 Coils

1. Acquire or prepare a hollow copper coil (5 mm × 5 mm) with 250 turns for generating a magnetic field. It yields a maximum of 500 A-turns at 2 A (the maximum allowed current of the amplifier used).
2. Mount the coil on the magnetic pole.
3. Current carrying coils can generate substantial heat (Joule Heating). Take appropriate steps to minimize heat generated (*see* **Note 10**).

3.2.3 Amplifier

1. A home-built amplifier can be used for driving the magnetic tweezers coil. The amplifier was built with four PA39 power amplifiers (Apex Microtechnology Corp). This amplifier can be controlled via a computer which allows very accurate control over the magnitude and nature of the current generated (step function, sine wave etc.) which is used for driving the coils.
2. Use the same amplifier also for controlling the LED illumination for fluorescence excitation.
3. Alternatively, commercially available, computer-controlled (or manually controlled), DC power sources can also be used. However, separate manual control of each part may hinder the synchronization of the entire setup via the computer.

3.3 Magnetic Tweezers Calibration

The magnetic forces can be increased or decreased by varying the driving current in the coils. The calibration is carried out via the viscous drag method as follows (*see* **Note 11**):

1. Suspend Dynal M270 magnetic microbeads in a high-viscosity liquid like Glycerol ($\eta = 1.5$ Pa s) in a chamber with a cover glass bottom. Glycerol is used since its viscosity is close to the viscosities expected in the intracellular environment.
2. Allow microbeads to settle down at the bottom of the chamber (~30 min).
3. Place the chamber with the microbeads on the microscope. The microbeads can be imaged in brightfield.
4. Dip the magnetic tweezers pole into the sample and move the tip in the field of view using the micromanipulator (*see* **Note 12**).
5. Turn on the current in the coil; microbeads should be seen to rapidly move towards the magnetic pole. Record microbeads' movement via a video camera connected to the microscope (*see* Fig. 6).
6. Track microbead movement under force over the entire video and calculate microbead velocity (see section on particle tracking).

7. Using the velocity and the known viscosity of the solution, calculate the force experienced by the microbeads from Stokes' law $\left(F_{drag} = 6\pi\eta r v\right)$ as a function of the distance from the pole tip (*see* **Note 11**).
8. Draw a force calibration curve (Fig. 3). The maximum magnetic force that can be exerted on a M270 microbead is in the range of ~1 nN at a distance of 10 μm from the pole tip and ~0.1 nN at 100 μm from the pole tip (*see* Fig. 3a). The magnetic force at a given distance from the tip can be increased up to saturation by increasing the current through the coil (*see* Fig. 3b).

3.4 Intracellular Magnetic Manipulation

For intracellular manipulation of phagosomes, the following steps were followed:

1. Incubate RAW 264.7 cells expressing Kmyr-GFP (*see* **Note 13**) overnight in a petri dish with cover glass bottom at 37 °C and 5% CO_2 atmosphere in DMEM culture medium with 10% Fetal Bovine Serum, 2 mM L-Glutamine and Pen-Strep.
2. Prior to the experiment the next morning, replace the culture medium with ice-cold DMEM medium (without FCS and l-glutamine but with 25 mM HEPES to maintain the pH) for 10 min in order to synchronize microbead internalization. Keep the cells on ice.

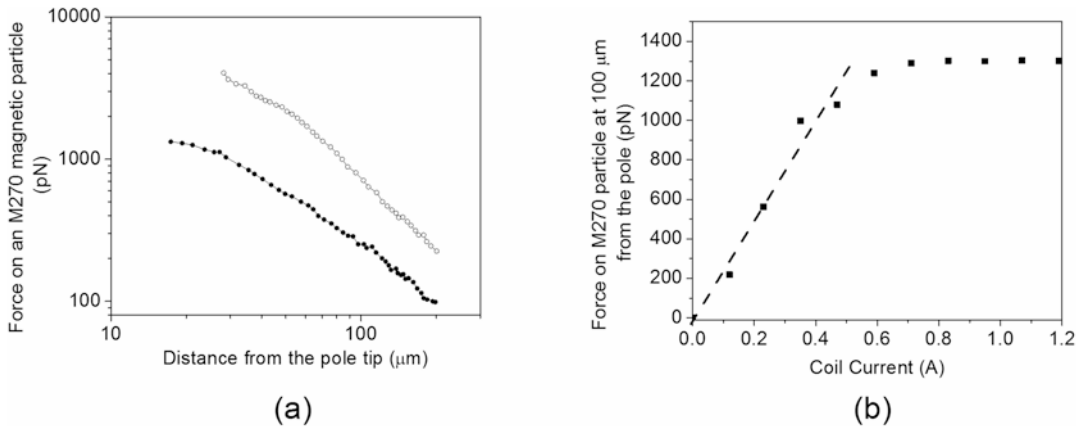

Fig. 3 Calibration of the single-pole magnetic tweezers. (**a**) Force applied on a Dynal M270 magnetic microbead as a function of distance between the pole and the microbead is shown for two different coil currents : 0.12A(*solid symbols*) and 0.23A(*open symbols*). The force experienced by the microbead increases as the distance between the microbead and the pole decreases. The force can also increases with increasing coil current (up to pole saturation). (**b**) The force applied on an M270 microbead by the magnetic pole for varying coil currents measured at a distance of 100 μm from the pole (the *dashed line* shows a linear fit for data-points prior to saturation). Reproduced from Shekhar et al. [11] with permission from Elsevier

3. Prepare a suspension of M270-SNARF microbeads in ice-cold medium (final concentration approx. 4×10^5 microbeads/ml of medium).
4. Add these microbeads to the cells while continuing to keep the cells on ice.
5. Allow beads to settle down and get attached to cells (~10 min).
6. Place the chamber on the prewarmed-microscope stage and allow the medium to warm up to 37 °C (temperature of the microscope incubator box).
7. Select a field of view with enough cells and microbeads. Also ensure that the field is not over-crowded with cells.
8. As the sample warms up, place the magnetic tweezers pole in the sample and carefully move the pole tip to the field of interest using the micromanipulator.
9. As the cells warm up, cells will start phagocytosing magnetic microbeads attached to them. Follow microbead internalization by observing the closure of the phagocytic cup using Kmyr-GFP (*see* **Note 13**) (Fig. 4).

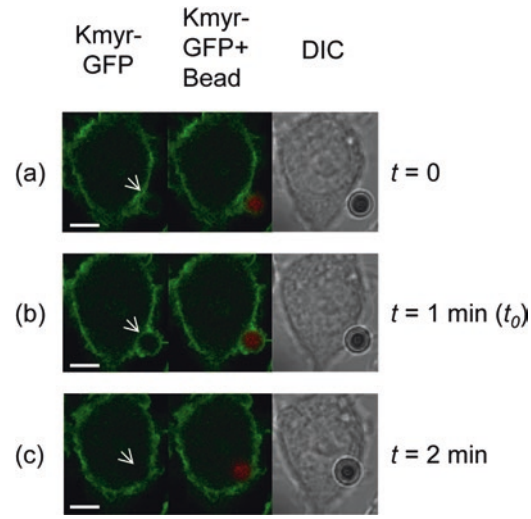

Fig. 4 Defining the time-point of microbead internalization (t_0). Time-lapse images of a RAW 264.7 cell expressing Kmyr-GFP (*green*) internalizing a M270-SNARF magnetic microbead (*red*). Successive images are a minute apart from each other. Figure shows (**a**) beginning of formation of the phagocytic cup (**b**) completion of the phagocytic cup at t_0 (**c**) the microbead moving inwards after internalization. At t_0, the Kmyr-GFP ring forms a uniform ring around the phagosome, indicating the closure of the phagocytic cup. Scale bar: 5 μm. Adapted from Shekhar et al. [11] with permission from Elsevier

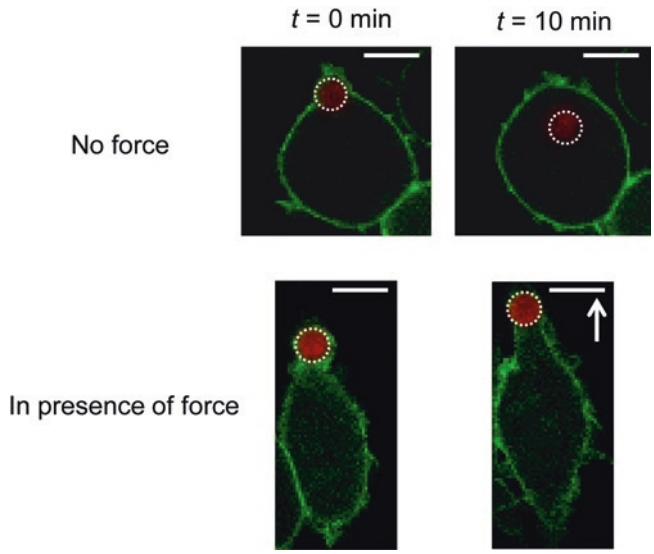

Fig. 5 Effect of force on a magnetic microbead containing phagosome. Time-lapse images showing the location of the magnetic microbead (*red, white dotted circle*) with respect to the plasma membrane of the cell (*green*) in absence (*top*) and presence (*bottom*) of an external magnetic force. The two frames are 10 min apart. In the absence of force (*top*), the microbead is transported inwards towards the cell nucleus. However, in the presence of force the microbead remains at the plasma membrane throughout the experiment (*bottom*). The white arrow denotes the direction in which the microbead experiences the magnetic force. Scale bar—5 μm. Reproduced from Shekhar et al. [11] with permission from Elsevier

10. As soon as the phagocytic cup gets closed, turn on the magnetic tweezers by switching on the current in the copper coil around the magnetic pole. The microbeads which are unattached (to cells) should immediately get attracted to the magnetic pole. The internalized magnetic microbeads should remain inside the cells (Fig. 5).
11. Vary the magnitude of the current in the coils to stall the centripetal transport of the magnetic microbead containing phagosome (Fig. 5). The phagosome transport through the cell can also be accelerated by changing the direction of the magnetic force by physical moving the tip to the opposite side with the micromanipulator.

3.5 Data Acquisition

In order to study the effect of intracellular manipulation of phagosomes on their acidification, the location of the phagosome (using brightfield imaging) and its pH (by fluorescence imaging) need to be simultaneously recorded. This requires simultaneous brightfield and widefield imaging, which is impractical to do in commercial microscopes. Since our experiment required fluorescence acquisition

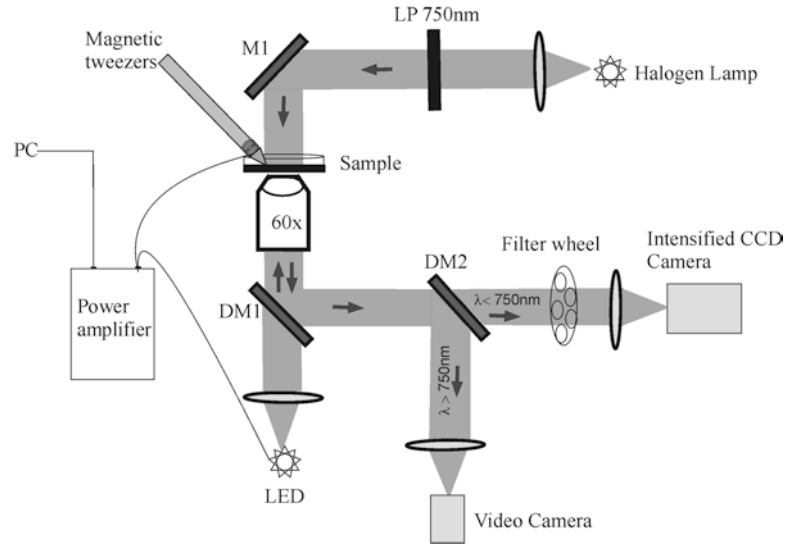

Fig. 6 Schematic representation of the customized optical setup as well the computer-controlled magnetic tweezers. The optical setup was assembled with a Nikon Eclipse TE 2000 microscope as the base. The setup is designed for simultaneous brightfield (in Near Infra-Red wavelength range) and fluorescence imaging. Fluorescence excitation is carried out by a computer-controlled LED. The filter wheel contains the appropriate band pass filters for ratiometric pH measurements. DM1 and DM2 are two dichroic mirrors, M1 is a mirror and LP 750 is a long pass 750 nm filter. A single-pole computer-controlled magnetic tweezers can also be seen placed on the microscope stage. Reproduced from Shekhar et al. [11] with permission from Elsevier

for pH-measurement, video acquisition would have to be temporarily stopped to allow fluorescence acquisition to prevent polluting the fluorescence image with white light (visible wavelengths) and overexposing the intensified CCD camera. To overcome these limitations, a home-built optical setup was designed.

1. Any commercially available widefield microscope can be used as a base (we used a Nikon Eclipse TE 2000). Figure 6 shows a schematic representation of the setup.
2. For fluorescence excitation, use an LED (wavelength 520–550 nm) instead of the mercury lamp. This also eliminates the need of an excitation filter in the filter cube. Remove the filter cube from the microscope.
3. The LED illumination is computer-controlled such that the LED is turned on only during acquisition, thus preventing needless illumination (and bleaching) of the fluorophores in between successive pH measurements (*see* **Note 14**).

4. Since the emission spectrum of the dye contains two pH-dependent peaks, pH-measurement require acquiring fluorescence images in two channels (Channel 1: 570–615 nm and Channel 2: 655–740 nm). Instead of using emission filters in the filter cube, use a computer-controlled filter-wheel instead. Place the band-pass emission filters for each channel in the filter wheel.
5. Acquire images in the two channels in series with the maximum integration time of 1 s per image using an intensified CCD (ICCD) camera. The thermally induced dark noise of the camera was reduced by internal cooling of the CCD chip to −20 °C.
6. Acquire fluorescence images once every 30 s to track phagosomal pH over time. The ratio of the microbead intensity in the two images is used to determine the phagosomal pH (*see* Subheading 3.6 for details).
7. Carry out brightfield imaging simultaneously with fluorescence imaging (*see* Fig. 7). Brightfield acquisition should be done at video rate (25 images per second) to allow monitoring small microbead displacements.

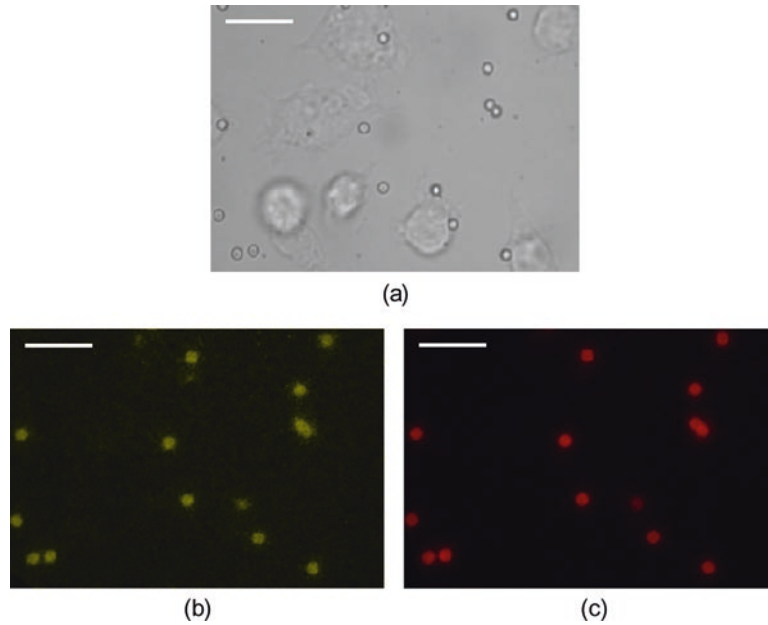

Fig. 7 Simultaneous brightfield and fluorescence imaging of phagocytosis. An example of (**a**) brightfield image acquired in Near-Infrared (NIR) wavelength region along with its complementary simultaneously acquired pH-dependent fluorescence images in (**b**) Channel 1 and (**c**) Channel 2. The ratio of microbead intensity in Channel 1 and Channel 2 is a measure of pH. Scale bar: 15 μm

8. Place a near infrared (NIR) filter (>750 nm) in front of the halogen lamp for brightfield imaging. Use the transmitted light as a light-source for brightfield imaging. Record using a video camera.
9. Use appropriate dichroic mirrors, to simultaneously record brightfield video in NIR wavelength using a video camera (WOTEC 902H) and fluorescently measure pH (once every 30 s) without interruption (*see* Fig. 8).

3.6 Data Analysis: Image Analysis and Particle Tracking

3.6.1 Image Analysis for pH-Measurement

Phagosomal pH is measured from two fluorescence images of beads (Channel 1: 570–615 nm and Channel 2: 655–740 nm). Although we used ImageJ, any other image analysis program can easily be used instead to carry out the following steps.

1. Carry out background correction on individual images in the two channels (Fig. 7b, c) using the rolling ball background subtraction algorithm to improve signal-to-noise.
2. Determine the microbead position in the Channel 1 image by applying an appropriate thresholding and subsequent particle (microbead) detection.
3. Be careful to select only spherical microbeads with an approximate expected size of 2.8μm (microbead size of M270 microbeads) for further analysis. In this way, microbead aggregates and/or other impurities are ignored.
4. Draw a circle of 2.8μm diameter at the center of microbead location and calculate the integrated fluorescence intensity of this circular area in both Channel 1 and Channel 2 images.

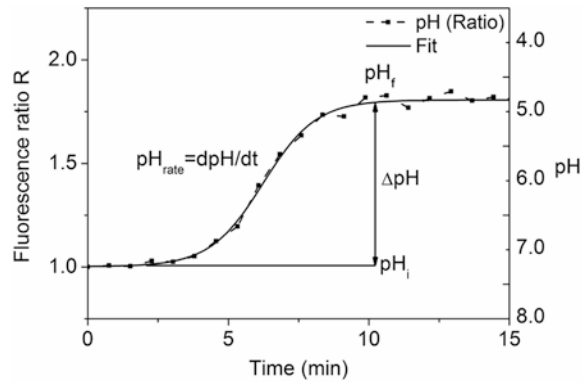

Fig. 8 Phagosomal acidification followed by a phagocytosed M270-SNARF microbead. Ratiometric measurement of phagosomal acidification by an M270-SNARF microbead upon its phagocytic uptake. As the phagosome acidifies, the ratio *R* increases (symbols, *dotted line*) and the pH decreases. The *thick line* represents the sigmoidal fit of the acidification curve (pH vs. time). The phagosome begins acidifying from an initial $pH = pH_i$ at a rate of acidification (pH_{rate}) to a final pH of pH_f at the completion of acidification

5. Determine the fluorescence ratio R for each microbead by dividing the integrated intensity of the microbead in Channel 1 by the integrated intensity of the microbead in Channel 2 i.e. $R = \frac{I_{ch1}}{I_{ch2}}$.
6. Normalize the fluorescence ratios R are such that the Ratio $R = 1$ at pH 7.
7. Determine the pH of the microbead using the calibration curve prepared earlier (*see* Fig. 1).
8. The above steps are repeated over the time-lapsed pH-images taken over the duration of the experiment.

3.6.2 Particle Tracking in Brightfield

A large number of particle tracking algorithms exist in order to accurately track the location of the microbeads. A custom-written program in LabVIEW based on pattern-recognition routines was used for particle tracking. Microbead tracks are also used for determining microbead velocities. Specifically the program functions as:

1. Identify the position of the microbead of interest manually in the first frame of the video.
2. Define a region of interest (ROI) around the microbead in which the program can attempt to find the microbead in the subsequent frame.
3. The ROI selection ensures that the microbead trajectory does not get polluted by a trajectory of any other microbeads in close vicinity.
4. Repeat above steps in an iterative fashion over successive frames to record microbead localization through the full duration of the video.
5. Determine the localization accuracy of your software by measuring the accuracy in tracking a microbead fixed on the glass surface. This will give the sensitivity of specific tracking method used.

4 Notes

1. Some magnetic beads can have appreciable autofluorescence in the visible wavelength range. This autofluorescence can pollute the signal coming from the pH-sensitive dye and can adversely affect ratiometric pH-measurement. It is therefore important to keep this in mind while choosing the beads.
2. Size and magnetic content of the particles (magnetic moment) are the two most important criteria while choosing magnetic microbeads. In this protocol, we have used amine-functionalized Dynal M270 microbeads. These are 2.8 μm in diameter,

adequately sized to follow phagosomal transport. Their magnetic content (volume magnetization = 11.5 kA/m) is also high enough to allow up to 1nN forces when placed in magnetic flux density gradients of a few tens of kT/m (typical gradients generated by single-pole magnetic tweezers).

3. Phenol red, which is the red-colored substance in cell culture media, is autofluorescent and will therefore interfere with the ratiometric pH-measurements. It is therefore important to use medium without phenol red during imaging. We are aware that some labs solve this problem by running their cell cultures in medium with phenol-red and they use non-phenol red medium during imaging. Nevertheless, we prefer to never expose cells to phenol red containing medium.
4. SNARF-4F can be dissolved in DMSO and divided into single-use volumes in microcentrifuge tubes. The dye can be concentrated under vacuum until all the solvent evaporates. The microcentrifuge tube can then be stored at −20 °C for up to 1 year at least.
5. pH-buffers for pH-calibrations can be prepared by mixing together 0.1 M citric acid and 0.2 M disodium hydrogen phosphate as follows [19]:

pH	0.2 M Na_2HPO_4	0.1 M citric acid
3.0	20.55 ml	79.45 ml
4.0	38.55 ml	61.45 ml
5.0	51.50 ml	48.50 ml
6.0	63.15 ml	36.85 ml
7.0	82.35 ml	17.65 ml
8.0	97.25 ml	2.75 ml

6. NHS is needed for the cross-linking reaction required for functionalizing the dye to the microbead surface. NHS increases the lifetime of the active intermediate formed from the EDC-COOH reaction, which reacts with the amine groups on the microbeads [20].
7. M270-SNARF beads that are functionalized only with fluorophores can be used for up to a month once prepared. However, once opsonised with the IgG, the beads should be used within 1 week. This is done to ensure that the antibodies on the beads remain active.
8. Over time, it will be noticed that the magnetic pole continues to stay magnetized even when the current has been turned off. When this occurs, the magnetic pole should be degaussed. This can be done by running a coil current with a decaying sinewave signal.

9. The tip of the magnetic tweezers pole can get rusted over time due to its frequent exposure to humidity. To prevent this, the tip can be kept dipped in edible oil during its storage. Ensure that the oil is completely removed prior to using the pole in cell experiments.
10. Electromagnets with high currents often produce substantial heat due to Joule Heating (Q). This in turn can cause heating of the cell culture medium close to the pole, leading to abnormal cellular behavior and even cell-death. If I is the current and N is the number of turns, $Q \propto I^2 \times N$. Therefore, Joule Heating increases with the square of the current but only linearly with N. Coil current should therefore be kept low to ensure this doesn't happen. In case heating occurs even at low current, adequate passive or active cooling strategies should be employed [15].
11. An object moving through a liquid experiences a retarding force against its motion due to the viscosity of the liquid [15]. This retarding force, called viscous drag, can be used for calibration. The viscous drag on a microbead is given by $\bar{F}_{\text{drag}} = \gamma \bar{v}$, where v is the velocity of the microbead and γ is the drag coefficient. For a spherical microbead of radius r, placed in a liquid of known viscosity η, the drag coefficient is given by $\gamma = 6\pi\eta r$. Under equilibrium conditions (~constant velocity), the magnitude of the drag force equals the magnitude of exerted magnetic force ($F_{\text{drag}} = F_{\text{mag}}$). By measuring the velocity of the microbead using video microscopy the position dependent magnetic force can be determined.
12. For magnetic tweezers calibration, make sure that the magnetic pole is as close as possible to the glass surface and only beads that stay in the same focal plane during their movement are used for calibration. This ensures that microbead velocity is not underestimated. It also helps with the automated tracking of the microbead afterwards.
13. Prior to manipulating a phagocytosed magnetic microbead, it is important to ensure that the microbead has been internalized and is not just sticking to the cell surface. Also, to compare parameters (e.g. beginning of acidification, end of acidification etc.) between different phagocytic events, it is essential to define an absolute time-point relative to which all other parameters can be set. We use the time-point of internalization (t_0), the time at which the microbead is completely enclosed in the phagocytic cup. For this we used a GFP-tagged polycationic probe Kmyr, a K-Ras-derived peptide that binds to anionic lipids [21]. Upon transfection and subsequent

expression, Kmyr-GFP is recruited to the plasma membrane of cells. Clear labeling of the plasma membrane by Kmyr-GFP ensures that formation and the closure of the phagocytic cup can be followed by observing a clear ring of Kmyr-GFP around the microbead (Fig. 4).

14. Photobleaching of SNARF-4F can sometimes cause a change in the ratio *R* (and therefore the measured pH). It can be partially prevented by using a low-intensity light source and reducing exposure as much as possible. In any case, it is important to always perform proper control experiments to take into account the effect of photobleaching. One way of easily doing this is to expose labeled beads to illumination of varying intensity and duration (at a fixed pH) and following the effect on ratio *R*.

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