

# Force Measurement of Living Professional Phagocytes of the Immune System

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1 Force measurement of living professional phagocytes of the immune system 2 3 Anna Mularski<sup>1,2,3</sup> and Florence Niedergang<sup>1,2,3</sup> 4 5 1. Inserm, U1016, Institut Cochin, Paris, France. 6 2. CNRS, UMR 8104, Paris, France. 7 3. Université Paris Descartes, Sorbonne Paris Cité, Paris, France. 8 9 10 Abstract 11 12 In higher organisms, the professional phagocytes of the immune system (dendritic cells, monocytes/macrophages and neutrophils), are responsible for pathogen clearance, the 13 14 development of immune responses via cytokine secretion and presentation of antigens 15 derived from the internalized material and the normal turnover and remodelling of tissues and disposal of dead cells. These functions rely on the ability of phagocytes to 16 17 migrate and adhere to sites of infection, dynamically probe their environments to make 18 contact with phagocytic targets, and to perform phagocytosis, a mechanism of 19 internalization of large particles, microorganisms and cellular debris for intracellular degradation. The cell generated forces that are necessary for the professional 20 phagocytes to act in their roles as 'first responders' of the immune system have been the 21 22 subject of mechanical studies in recent years. Methods of force measurement such as atomic force microscopy, traction force microscopy, micropipette aspiration, magnetic 23 and optical tweezers, and exciting new variants of these, have accompanied classical 24 25 biological methods to perform mechanical investigations of these highly dynamic 26 immune cells. 27 28 29 Introduction 30 31 Phagocytosis is the cellular mechanism of the internalization of large particles, microorganisms and cellular debris that can be up to several microns in size (1). Single 32 33 celled eukaryotes, such as amoebae and the slime mold, *Dictyostelium discoideum*, rely on phagocytosis for nutrition (2). In higher organisms, the professional phagocytes of 34 35 the immune system (dendritic cells, monocytes/macrophages and neutrophils), are responsible for pathogen clearance, the development of immune responses via cytokine 36 37 secretion and presentation of antigen derived from the internalized material and the 38 normal turnover and remodelling of tissues and disposal of dead cells (3). These cells

- 39 vary in size, morphology and the mode with which they carry out these vital immune
- 40 functions, however all of these cells perform phagocytosis with great efficiency.
- 41
- 42 The ability of phagocytes to migrate to sites of infection is instrumental in their role as
- 43 the 'first responders' of the immune system. Many immune cells migrate along chemical
- 44 gradients to encounter and engulf phagocytic targets (4-6). The signaling mechanisms
- 45 underlying migration have been determined in mesenchymal cells in particular (7, 8).
- 46 According to a classification based on morphology and molecular players, cells with
- 47 strong adhesion properties and protrusion activity at their leading edge are classified as

- 48 belonging to the mesenchymal migration mode, while cells with a less adhered
- 49 phenotype and a higher contractile activity have an amoeboid migration.
- 50

51 In mesenchymal cells, effective migration requires cells to attach to their surrounding 52 environment and generate traction against the material of which the surrounding 53 environment is composed. The cell then contracts, which exerts traction on the 54 underlying substrate generating strong cellular forces at the cell's leading edge. The 55 contraction is followed by the release of the cell's uropod and forward motion of the cell 56 is achieved (9-11). This mode of cellular migration is referred to as 'the towing mechanism'. Mesenchymal cells form strong focal adhesions, integrin-based structures 57 use adhere to the substrate and to serve as mechanosensors to sense local stiffness (12). 58 These adhesions with the substrate contain stress fibers that enable the large cellular 59 contractions required for their motility of mesenchymal cells (13). More recently 60 though, studies have appeared that investigated the mechanical mechanism of migration 61 and adhesion of the phagocytic cells of the immune system, which have long been 62 63 known to migrate at greater speeds with different (and shorter-lived) adhesive architecture than the larger, highly contractile mesenchymal cells. These studies have 64 65 revealed significant variation from earlier mechanistic studies of mesenchymal cells such as endothelial cells and fibroblasts. In addition, the physical properties of the cells' 66 67 environment also play an important role in defining the migration type of immune cells. This is especially true for dendritic cells, as described in a recent review (14). 68 69 70 Not all phagocytes migrate to the sites of infection, some reside in niches such that 71 phagocytic targets are encountered via the directed movement of lymph or blood flow, this can be a passive or stochastic event. (15-17). However, both migratory and non-72 migratory phagocytes also actively survey their environment with continuous, dynamic 73 74 probing to maximise encounters with phagocytic targets: phagocytic cells must be in 75 physical contact with their target as phagocytosis is initiated by the triggering of surface 76 receptors. Phagocytes probe their environments with actin driven protrusions, the 77 result of the dynamic formation and disassembly of actin networks directly under the 78 membrane surface. Flannagan et al. have demonstrated that these membrane 79 protrusions greatly enhance the frequency of receptor engagement with stationary or 80 randomly moving target particles (18). Cells that were unable to form these membrane protrusions failed to engage stationary particles (18, 19). Indeed, Nimmerjahn *et al.* (20) 81 82 demonstrated that membrane protrusions allow phagocytes to increase their scanning 83 radius by allowing access to spaces their cell bodies cannot access. 84

85 Once phagocyte and target are in physical contact, phagocytosis can be initiated by the 86 triggering of surface receptors for opsonins like immunoglobulins (IgG), in the case of the Fc receptor (FcR) mediated phagocytosis, or complement, in the case of integrin 87  $(\alpha M\beta 2)$  mediated phagocytosis. These opsonins coat the particulate antigen, labelling it 88 for internalisation and eventual degradation by phagocytosis. This process is shown 89 schematically in Figure 1. Phagocytosis can also be triggered by receptors that bind 90 91 directly to surface determinants of microorganisms, such as mannose receptors, scavenger receptors or Dectin-1 (21). Receptor triggering (Figure 1A) initiates a 92 transient burst of actin polymerization that forms the phagocytic cup (Figure 1B) and 93 provides the force with which membrane ruffles extend to engulf particulate matter. 94 95 Actin polymerisation continues at pseudopod tips, while depolymerisation of the actin at

96 the base of the phagocytic cup occurs, facilitating the membrane resupply required

97 (Figure 1C) (21). Closure of the phagosome occurs when membrane extensions meet,
98 promoted by actin and dynamin activities (Figure 1D), after which the phagosome is
99 internalised by the phagocyte (Figure 1E). After the cell achieves this significant
100 mechanical feat, the phagosome is degraded via series of fission and fusion events with
101 endocytic compartments.

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Figure 1: Schematic representation of phagocytosis. Phagocytosis begins with physical 104 contact between phagocyte and target (A) such that ligand-receptor binding can initiate 105 phagocytosis, beginning with a transient burst of actin polymerization that forms the 106 107 phagocytic cup (B) and provides the force with which membrane folds extend to engulf particulate matter. Actin polymerisation continues at pseudopod tips, while 108 depolymerisation of the actin at the base of the phagocytic cup occurs, facilitating the 109 membrane resupply required (C). Closure of the phagosome occurs when membrane 110 111 extensions meet (D), after which the phagosome is internalised by the phagocyte (E).

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113 The efficiency of the professional phagocytes in performing these vital immune

functions can be significantly reduced in patients with chronic illnesses (22, 23). This

115 can be due to microbes co-opting phagocytes to better invade host cells and/or to evade

immune responses (24). This is the case in particular after viral infections of

- 117 macrophages promoting the development of bacterial super-infections. Conversely,
- improper regulation of macrophage function in a chronic inflammatory environment hasbeen implicated in several diseases including cancer, rheumatoid arthritis and
- 120 atherosclerosis (25). Given this therapeutic significance, the professional phagocytes
- 121 have been the subject of intense study in medicine and cell biology. Significant progress
- 122 has been made in the elucidation of the signalling pathways governing the process, from
- 123 the triggering of phagocytic receptors to phagosome closure, internalisation and
- degradation (1, 26). More recently however, physical studies investigating the forces
- involved in the mechanical feats required for phagocytosis to occur have provided new
- avenues for investigation. Studies featuring force measurement of living phagocytic cells
- from traditional force measurement methods, such as atomic force microscopy,
   micropipette aspiration, magnetic and optical tweezers, as well less established and very
- 128 micropipette aspiration, magnetic and optical tweezers, as well less established and very 129 exciting methods such as traction force microscopy and new variants of these will form
- 130 the basis of this review.
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### Migration studies of the professional phagocytes

Cells are mechanically attached to neighboring cells and the extracellular matrix (ECM). 137 138 Forces generated by a cell are transmitted through these adhesions (tractions). Cellular tractions are very small (pN to nN range) and occur across small length scales (nm to 139 um range) making direct measurement of these forces difficult. A way around this 140 difficulty is to measure the deformations to flexible materials induced by cellular 141 tractions. Cellular tractions can thus be determined provided a substrate of well-defined 142 material properties. A broad family of techniques, operating on this principle, allow for 143 measurement of cellular tractions (27). The majority of migration studies of the 144 professional phagocytes have utilized a technique referred to as traction force 145 146 microscopy (TFM). As this technique is less commonly used than other more established 147 methods such as atomic force microscopy, or magnetic and optical tweezers, the basic 148 principles will be outlined here. Briefly, in standard (2D) TFM, small (~200 nm) 149 fluorescent beads are mixed into silicone or polyacrylamide substrates of known stiffness and coated with extracellular matrix proteins to facilitate cellular adhesion. The 150 151 fluorescent beads serve as fiduciary markers that can be tracked in space and time with optical microscopy. Typically, a TFM experiment requires optical imaging of the 152 distribution of beads at the substrate surface in a stressed state, and the imaging the 153 beads again in an unstressed state, usually after lysing the cell under observation. 154 155 Computational algorithms are then used to analyse the resulting images and to determine the displacement of the beads and the forces that would be required to cause 156 such displacement. The typical critique of standard TFM is that tractions are collected in 157 2D and therefore the method is 'blind' to forces exerted in the z direction. There are two 158 159 exciting variations to this approach that correct for this limitation, 2.5 and 3D TFM. In 2.5D TFM, the same experimental conditions apply as in standard TFM in that cells exert 160 forces on a planar, or 2D substrate, but bead displacement is observed in x, y and z. This 161 requires significantly more complex computation. The result though, is that cell 162 generated forces exerted on the substrate in x, y and z can be quantified (28-30). It is 163 important to note here that there is some confusion in the naming of the TFM variants. 164 In some studies, 2.5D TFM is referred to as '3D TFM' to reflect that forces are measured 165 in x, y and z, but as the cellular forces being exerted on a planar substrate, '2.5D TFM' is 166 more appropriate. 'Truly' 3D TFM, the measurement of cell generated forces in x, y and z 167 within a 3D substrate, is both technically and computationally demanding and 168 performed in very few laboratories in the world. The method requires the preparation 169 of porous 3D substrates of known and consistent material properties and very complex 170 171 computation for the tracking of fiduciary markers in 3D (31). To the best of our knowledge, 2.5D and 3D TFM have not yet been applied to the study of the professional 172 phagocytes of the immune system. As it is standard 2D TFM that is overwhelmingly 173 present in the literature of the professional phagocytes of the immune system, hereafter, 174 175 when TFM is discussed, it is standard 2D TFM that is being referred to. The differences between the techniques outlined in this section are summarised in Table 1 with 176 references for further reading and some links to open source code helpful to those who 177 wish to get started with TFM.' 178 179

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Technique	Measurement	Major strengths	Major limitations	Refs
Traction force	Cellular	Relatively simple	<ul> <li>Generally computationally</li> </ul>	(32-
microscopy (TFM)	traction forces	fabrication of substrates,	demanding, though some	37)
	in x, y plane	using standard lab supplies	ImageJ plugins are available	
		<ul> <li>Standard fluorescence</li> </ul>	that can be used to get started:	
		microscope for image	https://sites.google.com/site/q	
· · · ←		acquisition	<u>ingzongtseng/tfm (32)</u>	
			And some open source MATLAB	
			<mark>code:</mark>	
			<u>https://github.com/dkovari/TF</u>	
			<u>Matlab(33)</u>	
			<ul> <li>Requires lysis for</li> </ul>	
			determination of 'null force'	
			state	
2.5D TFM	Cellular	<ul> <li>Relatively simple</li> </ul>	<ul> <li>More computationally</li> </ul>	(28-
	traction forces	fabrication of substrates,	demanding than TFM	30)
	in x, y and z	using standard lab supplies	<ul> <li>Requires lysis for</li> </ul>	
			determination of 'null force'	
↓ ↓ ↓ ↓			state	
			<ul> <li>Requires confocal microscope</li> </ul>	
			for image acquisition	
3D TFM	Cellular	<ul> <li>Near native measurement</li> </ul>	<ul> <li>More computationally</li> </ul>	(31)
	traction forces	of traction forces	demanding than TFM and 2.5D	
	in x, y and z		TFM	
··· )	within a 3D		Requires lysis for	
	matrix		determination of 'null force'	
			state	
			Requires confocal microscope	
			for image acquisition	
			Preparation of substrates	
			more difficult than in TFM	(20
Micropillar	Cellular	• Forces are independent	Requires microfabrication and	(38,
	traction forces	for each pillar making	functionalisation of micropillar	39)
	ш х, у	computation relatively	substrate	
TIIII		simple		
		• Small tractions easier to		
		measure than in 1 FM		
		• Doesn't require lysis of		
		cells for determination of		
		nuil force state		

- **186** Table 1: Cellular traction force measurement techniques.
- 187

Smith *et al.* (36) and Jannat *et al.*(37) used TFM to study the migration of neutrophils. 188 Neutrophils are key players in the inflammatory response to injury and pathogens. They 189 are activated by chemoattractants leading to firm adhesion and rapid migration (20 190 191 µm/min). Neutrophils have also been known to turn quickly in response to chemical cues (13). The authors found that neutrophils achieve this motility not through frontal 192 towing but rather by 'tail contraction' or 'rearward-squeezing' where traction forces are 193 194 concentrated in the cells uropod and the cell is pushed forward through a squeezing mechanism, demonstrated to be dependent on ROCK and myosin activity. The authors 195 also found that the magnitude of neutrophil tractions were very small compared to 196 those generated by mesenchymal cells (consistent with the need to move quickly 197 198 towards phagocytic targets). 199

200 Ricart *et al.* (39) performed similar experiments on dendritic cells (DCs). DCs are

201 initiators of the adaptive immune response. They are stationed throughout the

202 periphery awaiting pathogen entry after which, they mature and migrate to lymph

nodes where they orchestrate lymphocyte activation. Given the extensive migration they 203 204 are required to undertake, it is very important that they can interpret external cues to migrate through different microenvironments. The authors used a micropillar array 205 206 detector, as they found that the traction forces exerted by DCs were too weak to reliably 207 quantify using TFM. PDMS micropillar arrays were fabricated with an effective stiffness of ~1.5 kPa. Tips of the micropillar were microcontact printed with fibronectin to 208 promote cell adhesion. DCs were allowed to adhere and exposed to chemokine gradients 209 210 and timelapse imaging revealed the deflection of micropillars due to cell migration. 211 Forces were calculated by multiplying the deflection of each individual micropillar by the spring constant of the micropillars. These experiments revealed that the maximal 212 stress was at the leading edge of the cell, an indicator of the towing model of migration, 213 214 with significantly less force exerted by DCs than neutrophils.

215

216 In two studies by Hind et al. (34, 35) macrophage migration was studied using TFM. Macrophages play an important role in the innate immune response by clearing 217 218 pathogens through phagocytosis and activating the adaptive immune response through cytokine production and antigen presentation. They need to be able to migrate to site of 219 220 infection. The TFM experiments of Hind et al. (34) revealed that the magnitude of macrophage tractions was dependent on the stiffness of the substrate. This result, from 221 222 experiments with substrates of four different stiffnesses, is important: macrophages migrate through very different microenvironments in the different parts of the 223 organism. Jannat *et al.* (37) observed the same trend with neutrophils though with 224 225 substrates of only two different stiffnesses. Like DCs, macrophages were found to 226 migrate using the towing mechanism of motility with the interesting difference that prior to macrophage contraction with which the towing mode of migration begins, 227 macrophages were found to extend and attach a pseudopod to the substrate. The 228 authors also demonstrate that macrophages are mechanoresponsive cells, hypothesing 229 230 that this ability may offer an advantage to cells that migrate through tissues of significantly different density. In a later publication from the same group, Hind et al. 231 232 (35) used TFM to study the motility of differently polarized macrophages. Macrophages can become polarized by cues in their environments resulting in functional changes. 233 234 Two main polarisations were obtained after in vitro treatment: pro-inflammatory M1 235 macrophages induced with IFNg and LPS, and anti-inflammatory M2 macrophages 236 treated with IL4. M0 macrophages were differentiated with M-CSF and not further 237 activated. The TFM experiments reported by Hind et al. demonstrate that M1 238 macrophages generate significantly less force than M0 macrophages, which in turn, 239 generate significantly less force than M2 macrophages. The same trend was observed for 240 motility. Related work demonstrated that macrophages use the amoeboid or the 241 mesenchymal migration modes depending of their subsets and activation status (40). 242 More importantly, as the authors state, differences in motility could serve as therapeutic targets in a number of diseases specifically associated with macrophage activity and 243 244 roles in the different phases of an inflammatory process. This sentiment could be extended to all of the phagocytes discussed here and these studies emphasise the 245 246 importance of studying the force generation of individual cell types: their motilities differ according to their functions. 247 248

- 249
- 250 Adhesion studies
- 251

- 252 Rather than focal adhesions, monocyte-derived macrophages form podosomes. These 253 are F-actin rich cone shaped structures that are  $\sim$ 600 nm in height, of submicron 254 diameter and form perpendicular to the substrate, surrounded by a ring of integrins and 255 other actin binding proteins (41). These adhesive structures have recently been investigated using atomic force microscopy (AFM). The versatility of this technique in 256 biological applications is long established and as such the operating principles will not 257 be discussed here. A recent and comprehensive overview of AFM in molecular and cell 258 259 biology applications can be found in Dufrêne et al. (42).
- 260

261 In an AFM and correlative fluorescence microscopy study, Labernadie et al. (43)

determined that podosomes are very dynamic, with a typical lifespan of  $\sim 10$  min with 262 oscillations in height and stiffness recorded throughout the lifetime of the structure. 263

Average podosome height and stiffness were accurately measured ( $578 \pm 209$  nm and 264

 $43.8 \pm 9.3$  kPa) and the authors found that these properties did not vary significantly 265

with changes in substrate. These AFM measurements of podosome height and stiffness 266

were performed, as is required with AFM, from the dorsal side. This is possible due to 267 268 the relative stiffness and significant height of the podosome structure when compared with the plasticity of the overlying plasma membrane. The authors acknowledge that the 269

determination of individual podosome forces is beyond the capability of a traditional 270 271 AFM setup, as this would require measurement from the ventral side. It seems that this limitation prompted the authors next research direction, as some years later, the 272

273 authors presented a novel, and complementary, AFM based method called 'protrusion

274 force microscopy' (PFM) to address this point (44). The differences in these two 275 approaches are shown in Table 2.

276

<b>Method</b>	Atomic force microscopy (AFM)	Protrusion force microscopy (PFM)
Experimental configuration		
Podosome size measurement	Dimensions of intracellular podosome structures measured from dorsal side	Dimensions of extracellular podosome structures measured from ventral side
<mark>Force</mark> measurement	Stiffness of podosomes determined by deflection of cantilever	Protrusion force calculated from deformations in formvar sheet to which cell is adhered

#### 277 Table 2: Differences between atomic force microscopy (AFM) and protrusion force

278

microscopy (PFM) for podosome studies.

- 279
- 280

281 PFM involves the use of AFM to measure local protrusions generated on a compliant

282 formvar sheet by macrophage podosomes. The forces generated by individual

podosomes were then estimated using a model that accounted for the mechanical 283

properties of the formvar sheet. It is interesting to note, that the name 'protrusion force 284

microscopy' was chosen by way of analogy with TFM. PFM is indeed complementary to 285

286 TFM as it addresses its major limitation: that forces in the z direction are not measured.

287 Given that the dominant adhesive structure in macrophages is the podosome (that

288 exerts force in the z direction), PFM has the potential to provide additional data on the 289

force generation of these cells, that until now have predominantly been investigated

290 using TFM. On application of their new method to living macrophages, Labernadie *et al.* found that single podosomes generate a protrusion force that increases with the

291

292 stiffness of the substrate - the hallmark of mechanosensing activity. The authors also 293 observed that these protrusions oscillate with a constant period and determined that

combined actomyosin contraction and actin polymerization were required for their 294

- 295 continuation.
- 296

#### 297 Probing the environment

#### 298 299

300 Cell membrane protrusions can be characterized according to shape. Veil like, several 301 micron broad protrusions are termed ruffles or lamellipodia. Tube like protrusions, 302 varying from a length of a few hundred nanometers, termed microspikes, to several microns long, termed filopodia. Kress et al. (45) used optical tweezers to trap opsonized 303 304 particles to study the interaction between phagocyte and phagocytic target. The authors used the optical trap to bring particles functionalized with opsonins to trigger different 305 phagocytic pathways into contact with cell membrane protrusions. They found that 306 overwhelmingly, the cells pulled the phagocytic target towards the cell body after ligand 307 308 receptor binding had occurred, regardless of opsonin type. Their results suggest that the earliest events in all phagocytic pathways share a common mechanism. 309

310

311 Several research groups have applied force measurement to the study of filopodia

function with respect to phagocytosis. Filopodia contain long bundles of parallel actin 312

filaments that penetrate deeply into the cytoplasm (46). Cojoc et al. (47) used optical 313

tweezers to measure the force that filopodia exert on a particle in a trap to be no greater 314

315 than 3 pN. In a complementary study using magnetic tweezers, Vonna et al.

316 demonstrated that as filopodia retract, they generate much larger forces over distances as great as 10 µm. The authors estimated the forces to be greater than 0.5 nN (48). To 317

318 study this retraction force in more detail, Kress et al. (49) used an optical trap to hold an

opsonized particle. The authors measured the force with which filopodia were retracted 319

320 back into the cell body, after receptor ligand binding had occurred. An interesting F-

321 actin-dependent stepwise retraction of filopodia was observed, suggesting that 322 molecular motors power the retraction. The authors also demonstrated that retraction

323 velocity was proportional to the counteracting force, from ~600 nm/s at low forces (<1

324 pN) to ~40 nm/s at higher forces (>15 pN) explaining the previous variation reported in

- 325 the literature (50).
- 326

## 327

328 329

At the maximal stage of pseudopod/membrane extension in phagocytosis, actin is 330 predominantly located in the tips, having cleared from the base of the phagocytic cup.

Cortical tension, membrane tension & internalisation

331 332 Once the target particle is fully surrounded, membrane fusion occurs between the

pseudopodia and the phagosome is sealed (separated from the plasma membrane). 333

Actin then quickly depolymerizes. It has been hypothesised that the availability of actin 334

is a rate-limiting step as it is recycled from the cup to the tips (51). Another parameter 335

336 upon which the successful engulfment and internalisation of the particle is dependent is

the availability of membrane to create the phagosome. Holevinski and Nelson (52) used 337

338 capacitance measurements to monitor particle uptake of human monocyte-derived 339 macrophages. Their results revealed that the surface of plasma membrane, rather than decreasing after engulfment of a large particle, increased. Bajno et al. (53) first 340 341 demonstrated that during phagocytosis, recycling vesicles accumulated at the phagocytic cup, suggesting that membrane extension results from internal membranes. 342 Various internal compartments were actually demonstrated to contribute to efficient 343 phagosome formation (54). Cortical tension measurements performed by Herant *et al.* 344 345 (55) on neutrophils provided another solution. In these experiments, neutrophils were 346 partially aspirated using a micropipette and the critical pressure difference at which the cell maintains an appendage of stationary lengths in the micropipette is related to the 347 cortical tension through Laplace's law. The experimental configuration, strengths and 348 349 limitations of such experiments are shown in Table 3. Herant et al. determined that in 350 addition to the trafficking of internal membranes to the phagocytic cup, the extent to 351 which expansion of the plasma membrane surface during phagocytosis takes place can 352 be explained by the release of 'spare' plasma membrane normally sequestered in a 353 reservoir of membrane folds, also used by the cell to probe its environment. Later, 354 internal compartments were revealed to play a role also as signalling platforms 355 important to regulate the local actin dynamics and depolymerisation that is necessary

- 356 for membrane extension (56).
- 357

358 Shown schematically in Figure 1, there remains some ambiguity about how phagosome closure and internalisation actually occur. Certainly, for fusion and internalisation to 359 360 take place, force generated by the actin driven protrusions must have directionality 361 (57). A contractile activity was identified by Evans *et al.* following pseudopod extension during the phagocytosis of yeast (58). More recently, a TFM study performed by Kovari 362 et al. (33) measured the tractions of the J774 macrophage cell line undergoing frustrated 363 phagocytosis. The authors were able to identify and quantify a contractile phase in cells 364 365 undergoing frustrated phagocytosis, corresponding to maximal traction forces exerted on the substrate, though it should be noted that frustrated phagocytosis is planar, and 366 367 therefore quite dissimilar from the 3D process. The activity of myosins (59-65) and a build-up of cortical tension (55, 60, 66, 67) have been suggested as the origin of a 368 369 contractile force required for internalisation. Several myosins are recruited at the site of 370 phagosome formation. MyosinK plays a critical role in efficient phagocytosis of large 371 particles in Dictyostelium discoideum (62). Myosin-II was described to be important for 372 CR3- more than FcR-mediated phagocytosis (65). In a later study by Herant et al. (66), 373 the authors couple their cortical tension measurements with mathematical modelling to 374 propose a model of FcR phagosome closure. They propose several possible models to 375 explain their experimental observations and find that the model that best fits their data 376 is based on a contractile flattening force with molecular motors (possibly, but not 377 necessarily myosins) that are anchored to the cell-particle interface, pulling down on the 378 actin cytoskeleton and pulling the target particle into the cell body.

379

In further work, Herant *et al.* (68) demonstrate that Zymosan triggered phagocytosis
occurs via a 'protrusive push' mechanism, with particles only internalised after an initial
outward push. They contrast this with the 'enveloping embrace' of FcR phagocytosis
(66), revealing that these two pathways display different force profiles. While the
precise origin of differing force profiles remains unclear, it has long been established
that the two most widely characterised phagocytic pathways, triggered by FcR and CR3
receptors, vary considerably in this respect (69). In both of these studies by Herant *et al.*

- 387 the cortical tension is considered the primary driver of inward motion of the target
- 388 particle, contrasting with the idea of a molecular motor driven inward pulling force. The 389 authors propose that it is the cortical tension driven tendency of a cell to round up, in
- authors propose that it is the cortical tension driven tendency of a cell to round up, inaddition to the strong adhesion between the target particle and the plasma membrane of
- addition to the strong adhesion between the target particle and the plasma membranethe phagocyte, that is the source of target motion into the cell. Later work is in good
- 392 agreement with this idea, but the biochemical basis of the contractile force remains
- unclear (33, 70). Recently though, using a Total Internal Reflection Fluorescence (TIRF)
- 394 Microscopy approach in living macrophages (26, 71), Marie-Anaïs *et al.* demonstrated
- 395 that the last scission step relies on the activity of the GTPase dynamin that acts in
- 396 concert with F-actin to mediate membrane scission and FcR-phagosome sealing (71).
- 397

Technique	Measurement	Force calculation	Major strengths	Major limitations	Refs
Micropipette aspiration	Cortical/ membrane tension	Critical pressure difference at which cell maintains an appendage of stationary length in the micropipette related to cortical tension through Laplace's law	<ul> <li>In house setup can be achieved at relatively low cost</li> <li>Standard microscopy</li> </ul>	• Experimental conditions far from native	(55, 66, 68, 72, 73)
Superresolved microparticle TFM	Cell generated forces during phagosome closure	Deformations of elastic phagocytic targets of quantified material properties can be used to infer surface traction forces	• Near native conditions achievable during measurement	Computationally demanding     Requires confocal microscope for image acquision     Preparation of microparticles requires specialized equipment	(74, 75)

## 398 Table 3: Methods for investigating phagosome closure.

399

- 400 More recently, Vorselen *et al.*(75) have made observations similar to Herant *et al.*, while presenting a new, and complementary, approach to study this critical phase of 401 internalisation of phagocytic targets. The authors describe a novel strategy, that they 402 name superresolved microparticle TFM, designed for studying ligand dependent cellular 403 interactions at high spatial resolution. Like PFM, this is a method that has its roots in 404 TFM and was developed to address certain limitations in the standard 2D configuration. 405 The method relies on the reliable fabrication of functionalised polyacrylamide spheres 406 407 of tunable stiffness. As a proof of concept, J774 murine macrophages were exposed to the spheres functionalised with IgG to trigger phagocytosis. Cell induced deformations 408 were observed and used to determine normal and shear stresses associated with 409 phagocytosis. The experimental configuration, strengths and limitations of the method 410 411 are shown in Table 3. The potential of this technique to be used to determine the role of various actin binding proteins involved in phagocytosis, particularly in the 412 internalisation/contractile phase, in near native conditions, is significant. 413
- 414 415

## 416 *Conclusions*

- 417
- The professional phagocytes of the immune system are responsible for many crucial
- 419 immune functions which rely on the ability of phagocytes to migrate and adhere to sites

420 of infection, dynamically probe their environments to make contact with phagocytic 421 targets, and to perform phagocytosis, a mechanism of internalization of large particles, microorganisms and cellular debris for intracellular degradation. These significant 422 mechanical feats are dependent on actin polymerization to provide the force that drives 423 424 membrane deformation. The role of the actin cytoskeleton at key stages of these 425 processes has been extensively studied, particularly with regard to migration, 426 membrane protrusions and pseudopod extension. In recent years, researchers of 427 phagocytosis have coupled traditional methods in medicine and cell biology with methods of force measurement, such as atomic and traction force microscopies, optical 428 429 and magnetic tweezers and micropipette aspiration to perform mechanistic studies of phagocytosis, providing great insight into this essential immune process. There is a 430 great deal that remains unknown about phagocytosis though, particularly around 431 phagosome sealing and particle internalisation. Both the origin of the contractile force 432 and its trigger are undetermined, though myosins, membrane and cortical tension 433 feature in models that have been proposed. Further studies of the forces involved in this 434 435 dramatic process present an exciting path forward, particularly as new variants of more established techniques are developed, such as in the work of Vorselen et al. (75) and 436 Labernadie et al. (44). It is the design of such live cell experimental methodologies that 437 438 along with classical biological methods, may in time, be able to reveal the true variety 439 and complexity of this and other processes in the future. 440 441 **Acknowledgements** 442 443 The authors would like to thank Sophie Echène for the lovely illustrations in this review. 444 This work was supported by grants from Centre National de la Recherche Scientifique 445 (CNRS), Institut National de la Santé et de la Recherche Médicale (Inserm), Université 446 Paris Descartes and Agence Nationale de la Recherche (ANR 16-CE13-0007-01) that 447 supported AM for a post-doctoral position. 448 449 450 Declaration 451 452 The authors declare no conflict of interest. 453 454 References 455 456 457 Ronald S. Flannagan VJ, and Sergio Grinstein. The Cell Biology of Phagocytosis. 1. Annual Review of Pathology: Mechanisms of Disease. 2012;7(1):61-98. 458 459 Niedergang F. Phagocytosis. Encyclopedia of Cell Biology. Waltham: Academic Press; 2. 460 2016. p. 751-7. 461 3. Poon IKH, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic 462 biology and therapeutic potential. Nat Rev Immunol. 2014;14(3):166-80. 463 Bloes DA, Kretschmer D, Peschel A. Enemy attraction: bacterial agonists for leukocyte 4. chemotaxis receptors. Nat Rev Microbiol. 2015;13(2):95-104. 464 465 5. Heit B, Liu L, Colarusso P, Puri KD, Kubes P. PI3K accelerates, but is not required for, 466 neutrophil chemotaxis to fMLP. Journal of Cell Science. 2008;121(2):205. 467 6. Devosse T, Guillabert A, Haene N, Berton A, De Nadai P, Noel S, et al. Formyl Peptide

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