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HAL Authorization

1 ***Force measurement of living professional phagocytes of the immune system***

2
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8 9 10 ***Abstract***

11
12 In higher organisms, the professional phagocytes of the immune system (dendritic cells,
13 monocytes/macrophages and neutrophils), are responsible for pathogen clearance, the
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
16 tissues and disposal of dead cells. These functions rely on the ability of phagocytes to
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
20 degradation. The cell generated forces that are necessary for the professional
21 phagocytes to act in their roles as ‘first responders’ of the immune system have been the
22 subject of mechanical studies in recent years. Methods of force measurement such as
23 atomic force microscopy, traction force microscopy, micropipette aspiration, magnetic
24 and optical tweezers, and exciting new variants of these, have accompanied classical
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,
32 microorganisms and cellular debris that can be up to several microns in size (1). Single
33 celled eukaryotes, such as amoebae and the slime mold, *Dictyostelium discoideum*, rely
34 on phagocytosis for nutrition (2). In higher organisms, the professional phagocytes of
35 the immune system (dendritic cells, monocytes/macrophages and neutrophils), are
36 responsible for pathogen clearance, the development of immune responses via cytokine
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

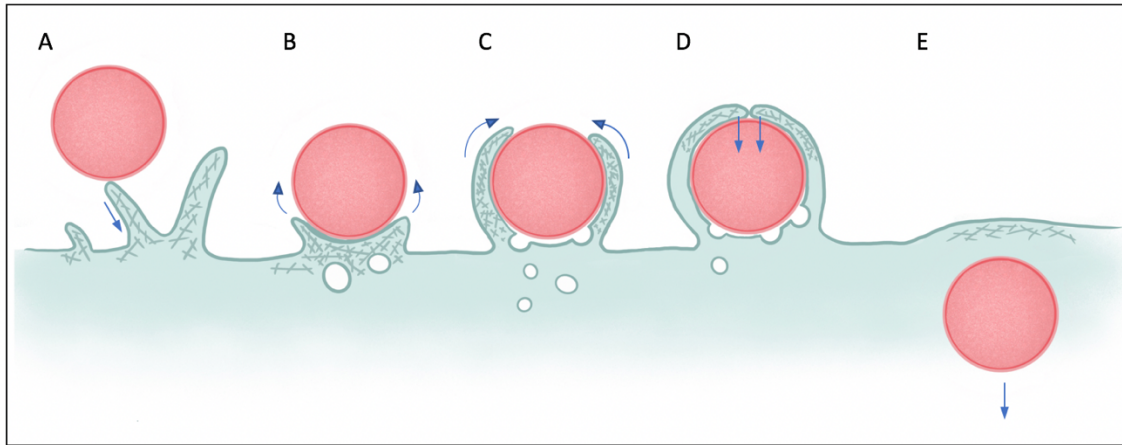
48 belonging to the mesenchymal migration mode, while cells with a less adhered
49 phenotype and a higher contractile activity have an amoeboid migration.

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
57 mechanism'. Mesenchymal cells form strong focal adhesions, integrin-based structures
58 use adhere to the substrate and to serve as mechanosensors to sense local stiffness (12).
59 These adhesions with the substrate contain stress fibers that enable the large cellular
60 contractions required for their motility of mesenchymal cells (13). More recently
61 though, studies have appeared that investigated the mechanical mechanism of migration
62 and adhesion of the phagocytic cells of the immune system, which have long been
63 known to migrate at greater speeds with different (and shorter-lived) adhesive
64 architecture than the larger, highly contractile mesenchymal cells. These studies have
65 revealed significant variation from earlier mechanistic studies of mesenchymal cells
66 such as endothelial cells and fibroblasts. In addition, the physical properties of the cells'
67 environment also play an important role in defining the migration type of immune cells.
68 This is especially true for dendritic cells, as described in a recent review (14).

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,
72 this can be a passive or stochastic event. (15-17). However, both migratory and non-
73 migratory phagocytes also actively survey their environment with continuous, dynamic
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
81 protrusions failed to engage stationary particles (18, 19). Indeed, Nimmerjahn *et al.* (20)
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
87 the Fc receptor (FcR) mediated phagocytosis, or complement, in the case of integrin
88 ($\alpha M\beta 2$) mediated phagocytosis. These opsonins coat the particulate antigen, labelling it
89 for internalisation and eventual degradation by phagocytosis. This process is shown
90 schematically in Figure 1. Phagocytosis can also be triggered by receptors that bind
91 directly to surface determinants of microorganisms, such as mannose receptors,
92 scavenger receptors or Dectin-1 (21). Receptor triggering (Figure 1A) initiates a
93 transient burst of actin polymerization that forms the phagocytic cup (Figure 1B) and
94 provides the force with which membrane ruffles extend to engulf particulate matter.
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.
102



103
104 Figure 1: Schematic representation of phagocytosis. Phagocytosis begins with physical
105 contact between phagocyte and target (A) such that ligand-receptor binding can initiate
106 phagocytosis, beginning with a transient burst of actin polymerization that forms the
107 phagocytic cup (B) and provides the force with which membrane folds extend to engulf
108 particulate matter. Actin polymerisation continues at pseudopod tips, while
109 depolymerisation of the actin at the base of the phagocytic cup occurs, facilitating the
110 membrane resupply required (C). Closure of the phagosome occurs when membrane
111 extensions meet (D), after which the phagosome is internalised by the phagocyte (E).
112

113 The efficiency of the professional phagocytes in performing these vital immune
114 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
116 immune responses (24). This is the case in particular after viral infections of
117 macrophages promoting the development of bacterial super-infections. Conversely,
118 improper regulation of macrophage function in a chronic inflammatory environment has
119 been 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
124 degradation (1, 26). More recently however, physical studies investigating the forces
125 involved in the mechanical feats required for phagocytosis to occur have provided new
126 avenues for investigation. Studies featuring force measurement of living phagocytic cells
127 from traditional force measurement methods, such as atomic force microscopy,
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.

131
132
133
134

135 *Migration studies of the professional phagocytes*

136

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



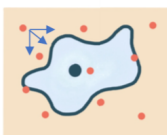

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Technique	Measurement	Major strengths	Major limitations	Refs
Traction force microscopy (TFM) 	Cellular traction forces in x, y plane	<ul style="list-style-type: none"> • Relatively simple fabrication of substrates, using standard lab supplies • Standard fluorescence microscope for image acquisition 	<ul style="list-style-type: none"> • Generally computationally demanding, though some ImageJ plugins are available that can be used to get started: https://sites.google.com/site/qingzongtseng/tfm (32) • And some open source MATLAB code: https://github.com/dkovari/TFMatlab(33) • Requires lysis for determination of 'null force' state 	(32-37)
2.5D TFM 	Cellular traction forces in x, y and z	<ul style="list-style-type: none"> • Relatively simple fabrication of substrates, using standard lab supplies 	<ul style="list-style-type: none"> • More computationally demanding than TFM • Requires lysis for determination of 'null force' state • Requires confocal microscope for image acquisition 	(28-30)
3D TFM 	Cellular traction forces in x, y and z within a 3D matrix	<ul style="list-style-type: none"> • Near native measurement of traction forces 	<ul style="list-style-type: none"> • More computationally demanding than TFM and 2.5D TFM • Requires lysis for determination of 'null force' state • Requires confocal microscope for image acquisition • Preparation of substrates more difficult than in TFM 	(31)
Micropillar 	Cellular traction forces in x, y	<ul style="list-style-type: none"> • Forces are independent for each pillar making computation relatively simple • Small tractions easier to measure than in TFM • Doesn't require lysis of cells for determination of 'null force' state 	<ul style="list-style-type: none"> • Requires microfabrication and functionalisation of micropillar substrate 	(38, 39)

186 Table 1: Cellular traction force measurement techniques.

187

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

203 nodes where they orchestrate lymphocyte activation. Given the extensive migration they
204 are required to undertake, it is very important that they can interpret external cues to
205 migrate through different microenvironments. The authors used a micropillar array
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
208 of ~ 1.5 kPa. Tips of the micropillar were microcontact printed with fibronectin to
209 promote cell adhesion. DCs were allowed to adhere and exposed to chemokine gradients
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
212 the spring constant of the micropillars. These experiments revealed that the maximal
213 stress was at the leading edge of the cell, an indicator of the towing model of migration,
214 with significantly less force exerted by DCs than neutrophils.

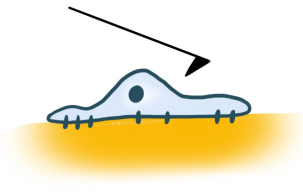
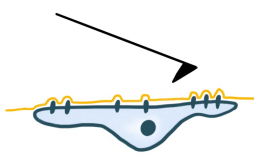
215
216 In two studies by Hind *et al.* (34, 35) macrophage migration was studied using TFM.
217 Macrophages play an important role in the innate immune response by clearing
218 pathogens through phagocytosis and activating the adaptive immune response through
219 cytokine production and antigen presentation. They need to be able to migrate to site of
220 infection. The TFM experiments of Hind *et al.* (34) revealed that the magnitude of
221 macrophage tractions was dependent on the stiffness of the substrate. This result, from
222 experiments with substrates of four different stiffnesses, is important: macrophages
223 migrate through very different microenvironments in the different parts of the
224 organism. Jannat *et al.* (37) observed the same trend with neutrophils though with
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
227 prior to macrophage contraction with which the towing mode of migration begins,
228 macrophages were found to extend and attach a pseudopod to the substrate. The
229 authors also demonstrate that macrophages are mechanoresponsive cells, hypothesing
230 that this ability may offer an advantage to cells that migrate through tissues of
231 significantly different density. In a later publication from the same group, Hind *et al.*
232 (35) used TFM to study the motility of differently polarized macrophages. Macrophages
233 can become polarized by cues in their environments resulting in functional changes.
234 Two main polarisations were obtained after *in vitro* treatment: pro-inflammatory M1
235 macrophages induced with IFN γ 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
243 targets in a number of diseases specifically associated with macrophage activity and
244 roles in the different phases of an inflammatory process. This sentiment could be
245 extended to all of the phagocytes discussed here and these studies emphasise the
246 importance of studying the force generation of individual cell types: their motilities
247 differ according to their functions.

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 ~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
 256 investigated using atomic force microscopy (AFM). The versatility of this technique in
 257 biological applications is long established and as such the operating principles will not
 258 be discussed here. A recent and comprehensive overview of AFM in molecular and cell
 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)
 262 determined that podosomes are very dynamic, with a typical lifespan of ~10 min with
 263 oscillations in height and stiffness recorded throughout the lifetime of the structure.
 264 Average podosome height and stiffness were accurately measured (578 ± 209 nm and
 265 43.8 ± 9.3 kPa) and the authors found that these properties did not vary significantly
 266 with changes in substrate. These AFM measurements of podosome height and stiffness
 267 were performed, as is required with AFM, from the dorsal side. This is possible due to
 268 the relative stiffness and significant height of the podosome structure when compared
 269 with the plasticity of the overlying plasma membrane. The authors acknowledge that the
 270 determination of individual podosome forces is beyond the capability of a traditional
 271 AFM setup, as this would require measurement from the ventral side. It seems that this
 272 limitation prompted the authors next research direction, as some years later, the
 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

Method	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
Force 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
 283 podosomes were then estimated using a model that accounted for the mechanical
 284 properties of the formvar sheet. It is interesting to note, that the name ‘protrusion force
 285 microscopy’ was chosen by way of analogy with TFM. PFM is indeed complementary to
 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.*
291 found that single podosomes generate a protrusion force that increases with the
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
294 combined actomyosin contraction and actin polymerization were required for their
295 continuation.

296
297

298 ***Probing the environment***

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
303 microns long, termed filopodia. Kress *et al.* (45) used optical tweezers to trap opsonized
304 particles to study the interaction between phagocyte and phagocytic target. The authors
305 used the optical trap to bring particles functionalized with opsonins to trigger different
306 phagocytic pathways into contact with cell membrane protrusions. They found that
307 overwhelmingly, the cells pulled the phagocytic target towards the cell body after ligand
308 receptor binding had occurred, regardless of opsonin type. Their results suggest that the
309 earliest events in all phagocytic pathways share a common mechanism.

310

311 Several research groups have applied force measurement to the study of filopodia
312 function with respect to phagocytosis. Filopodia contain long bundles of parallel actin
313 filaments that penetrate deeply into the cytoplasm (46). Cojoc *et al.* (47) used optical
314 tweezers to measure the force that filopodia exert on a particle in a trap to be no greater
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
317 as great as 10 μm . The authors estimated the forces to be greater than 0.5 nN (48). To
318 study this retraction force in more detail, Kress *et al.* (49) used an optical trap to hold an
319 opsonized particle. The authors measured the force with which filopodia were retracted
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 ***Cortical tension, membrane tension & internalisation***

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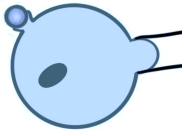

330 At the maximal stage of pseudopod/membrane extension in phagocytosis, actin is
331 predominantly located in the tips, having cleared from the base of the phagocytic cup.
332 Once the target particle is fully surrounded, membrane fusion occurs between the
333 pseudopodia and the phagosome is sealed (separated from the plasma membrane).
334 Actin then quickly depolymerizes. It has been hypothesised that the availability of actin
335 is a rate-limiting step as it is recycled from the cup to the tips (51). Another parameter
336 upon which the successful engulfment and internalisation of the particle is dependent is
337 the availability of membrane to create the phagosome. Holevinski and Nelson (52) used

338 capacitance measurements to monitor particle uptake of human monocyte-derived
339 macrophages. Their results revealed that the surface of plasma membrane, rather than
340 decreasing after engulfment of a large particle, increased. Bajno *et al.* (53) first
341 demonstrated that during phagocytosis, recycling vesicles accumulated at the
342 phagocytic cup, suggesting that membrane extension results from internal membranes.
343 Various internal compartments were actually demonstrated to contribute to efficient
344 phagosome formation (54). Cortical tension measurements performed by Herant *et al.*
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
347 cell maintains an appendage of stationary lengths in the micropipette is related to the
348 cortical tension through Laplace's law. The experimental configuration, strengths and
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
359 closure and internalisation actually occur. Certainly, for fusion and internalisation to
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
362 during the phagocytosis of yeast (58). More recently, a TFM study performed by Kovari
363 *et al.* (33) measured the tractions of the J774 macrophage cell line undergoing frustrated
364 phagocytosis. The authors were able to identify and quantify a contractile phase in cells
365 undergoing frustrated phagocytosis, corresponding to maximal traction forces exerted
366 on the substrate, though it should be noted that frustrated phagocytosis is planar, and
367 therefore quite dissimilar from the 3D process. The activity of myosins (59-65) and a
368 build-up of cortical tension (55, 60, 66, 67) have been suggested as the origin of a
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
380 In further work, Herant *et al.* (68) demonstrate that Zymosan triggered phagocytosis
381 occurs via a 'protrusive push' mechanism, with particles only internalised after an initial
382 outward push. They contrast this with the 'enveloping embrace' of FcR phagocytosis
383 (66), revealing that these two pathways display different force profiles. While the
384 precise origin of differing force profiles remains unclear, it has long been established
385 that the two most widely characterised phagocytic pathways, triggered by FcR and CR3
386 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
 390 addition to the strong adhesion between the target particle and the plasma membrane of
 391 the 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
 393 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 style="list-style-type: none"> • In house setup can be achieved at relatively low cost • Standard microscopy 	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • Near native conditions achievable during measurement 	<ul style="list-style-type: none"> • Computationally demanding • Requires confocal microscope for image acquisition • 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
 401 presenting a new, and complementary, approach to study this critical phase of
 402 internalisation of phagocytic targets. The authors describe a novel strategy, that they
 403 name superresolved microparticle TFM, designed for studying ligand dependent cellular
 404 interactions at high spatial resolution. Like PFM, this is a method that has its roots in
 405 TFM and was developed to address certain limitations in the standard 2D configuration.
 406 The method relies on the reliable fabrication of functionalised polyacrylamide spheres
 407 of tunable stiffness. As a proof of concept, J774 murine macrophages were exposed to
 408 the spheres functionalised with IgG to trigger phagocytosis. Cell induced deformations
 409 were observed and used to determine normal and shear stresses associated with
 410 phagocytosis. The experimental configuration, strengths and limitations of the method
 411 are shown in Table 3. The potential of this technique to be used to determine the role of
 412 various actin binding proteins involved in phagocytosis, particularly in the
 413 internalisation/contractile phase, in near native conditions, is significant.
 414

415
 416 **Conclusions**

417
 418 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,
422 microorganisms and cellular debris for intracellular degradation. These significant
423 mechanical feats are dependent on actin polymerization to provide the force that drives
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
428 methods of force measurement, such as atomic and traction force microscopies, optical
429 and magnetic tweezers and micropipette aspiration to perform mechanistic studies of
430 phagocytosis, providing great insight into this essential immune process. There is a
431 great deal that remains unknown about phagocytosis though, particularly around
432 phagosome sealing and particle internalisation. Both the origin of the contractile force
433 and its trigger are undetermined, though myosins, membrane and cortical tension
434 feature in models that have been proposed. Further studies of the forces involved in this
435 dramatic process present an exciting path forward, particularly as new variants of more
436 established techniques are developed, such as in the work of Vorselen *et al.* (75) and
437 Labernadie *et al.* (44). It is the design of such live cell experimental methodologies that
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

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442

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448

449

450 **Declaration**

451

452 The authors declare no conflict of interest.

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454

455 **References**

456

- 457 1. Ronald S. Flannagan VJ, and Sergio Grinstein. The Cell Biology of Phagocytosis.
458 Annual Review of Pathology: Mechanisms of Disease. 2012;7(1):61-98.
- 459 2. Niedergang F. Phagocytosis. Encyclopedia of Cell Biology. Waltham: Academic Press;
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 4. Bloes DA, Kretschmer D, Peschel A. Enemy attraction: bacterial agonists for leukocyte
464 chemotaxis receptors. Nat Rev Microbiol. 2015;13(2):95-104.
- 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
468 Receptor-Like 2 Is Expressed and Functional in Plasmacytoid Dendritic Cells, Tissue-Specific

469 Macrophage Subpopulations, and Eosinophils. *The Journal of Immunology*.
470 2009;182(8):4974.

471 7. Ridley AJ. Rho proteins, PI 3-kinases, and monocyte/macrophage motility. *FEBS*
472 *Letters*. 2001;498(2):168-71.

473 8. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell
474 Migration: Integrating Signals from Front to Back. *Science*. 2003;302(5651):1704.

475 9. Pelham RJ, Wang Y-l. Cell locomotion and focal adhesions are regulated by
476 substrate flexibility. *Proc Natl Acad Sci U S A*. 1997;94(25):13661-5.

477 10. Reinhart-King CA. Chapter 3 Endothelial Cell Adhesion and Migration. *Methods in*
478 *Enzymology*. 443: Academic Press; 2008. p. 45-64.

479 11. Reinhart-King CA, Dembo M, Hammer DA. The Dynamics and Mechanics of
480 Endothelial Cell Spreading. *Biophys J*. 2005;89(1):676-89.

481 12. Lo C-M, Wang H-B, Dembo M, Wang Y-l. Cell Movement Is Guided by the Rigidity of
482 the Substrate. *Biophys J*. 2000;79(1):144-52.

483 13. Lauffenburger DA, Linderman JJ. Receptors. [electronic resource] : models for
484 binding, trafficking, and signaling: Oxford University Press; 1993.

485 14. Bidan CM, Fratzl M, Coullomb A, Moreau P, Lombard AH, Wang I, et al. Magneto-
486 active substrates for local mechanical stimulation of living cells. *Sci Rep*. 2018;8(1):1464.

487 15. Kohyama M, Ise W, Edelson BT, Wilker PR, Hildner K, Mejia C, et al. Role for Spi-C in
488 the development of red pulp macrophages and splenic iron homeostasis. *Nature*.
489 2009;457(7227):318-21.

490 16. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease.
491 *Liver International*. 2006;26(10):1175-86.

492 17. Gerner Michael Y, Torabi-Parizi P, Germain Ronald N. Strategically Localized Dendritic
493 Cells Promote Rapid T Cell Responses to Lymph-Borne Particulate Antigens. *Immunity*.
494 2015;42(1):172-85.

495 18. Flannagan RS, Harrison RE, Yip CM, Jaqaman K, Grinstein S. Dynamic macrophage
496 "probing" is required for the efficient capture of phagocytic targets. *The Journal of Cell*
497 *Biology*. 2010;191(6):1205.

498 19. Miller YI, Chang M-K, Funk CD, Feramisco JR, Witztum JL. 12/15-Lipoxygenase
499 Translocation Enhances Site-specific Actin Polymerization in Macrophages Phagocytosing
500 Apoptotic Cells. *Journal of Biological Chemistry*. 2001;276(22):19431-9.

501 20. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting Microglial Cells Are Highly Dynamic
502 Surveillants of Brain Parenchyma in Vivo. *Science*. 2005;308(5726):1314.

503 21. Jubrail J, Montauban K, Regis-Burgel P, Kurian N, Niedergang F. Characterisation of
504 defective phagocytosis by chronic obstructive pulmonary disease alveolar macrophages.
505 *European Respiratory Journal*. 2016;48(suppl 60).

506 22. Tabas I, Glass CK. Anti-Inflammatory Therapy in Chronic Disease: Challenges and
507 Opportunities. *Science*. 2013;339(6116):166.

508 23. Jubrail JK, Nisha; Niedergang, Florence. Macrophage phagocytosis cracking the defect
509 code in COPD. *Biomedical Journal*. 2017;In press.

510 24. Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and
511 bacterial evasion strategies. *Nat Rev Microbiol*. 2009;7(5):355-66.

512 25. Pollard JW. Trophic macrophages in development and disease. *Nature Reviews*
513 *Immunology*. 2009;9:259.

514 26. Mularski A M-AF, Mazzolini J and Niedergang, F. Observing frustrated phagocytosis
515 and phagosome formation and closure using total internal reflection fluorescence

516 microscopy (TIRFM). In: Rousselet G, editor. *Methods in Molecular Biology*. In press:
517 Springer; 2018.

518 27. Polacheck WJ, Chen CS. Measuring cell-generated forces: a guide to the available
519 tools. *Nat Meth*. 2016;13(5):415-23.

520 28. Legant WR, Choi CK, Miller JS, Shao L, Gao L, Betzig E, et al. Multidimensional traction
521 force microscopy reveals out-of-plane rotational moments about focal adhesions. *Proc Natl*
522 *Acad Sci U S A*. 2013;110(3):881-6.

523 29. Toyjanova J, Bar-Kochba E, López-Fagundo C, Reichner J, Hoffman-Kim D, Franck C.
524 High Resolution, Large Deformation 3D Traction Force Microscopy. *PLOS ONE*.
525 2014;9(4):e90976.

526 30. Maskarinec SA, Franck C, Tirrell DA, Ravichandran G. Quantifying cellular traction
527 forces in three dimensions. *Proc Natl Acad Sci U S A*. 2009;106(52):22108-13.

528 31. Legant WR, Miller JS, Blakely BL, Cohen DM, Genin GM, Chen CS. Measurement of
529 mechanical tractions exerted by cells in three-dimensional matrices. *Nat Meth*.
530 2010;7(12):969-71.

531 32. Martiel J-L, Leal A, Kurzawa L, Balland M, Wang I, Vignaud T, et al. Measurement of
532 cell traction forces with ImageJ. *Methods in Cell Biology*. 2015;125:269-87.

533 33. Kovari DT, Wei W, Chang P, Toro J-S, Beach RF, Chambers D, et al. Frustrated
534 Phagocytic Spreading of J774A-1 Macrophages Ends in Myosin II-Dependent Contraction.
535 *Biophys J*. 2016;111(12):2698-710.

536 34. Hind LE, Dembo M, Hammer DA. Macrophage motility is driven by frontal-towing
537 with a force magnitude dependent on substrate stiffness. *Integrative Biology*. 2015;7(4):447-
538 53.

539 35. Hind LE, Lurier EB, Dembo M, Spiller KL, Hammer DA. Effect of M1–M2 Polarization
540 on the Motility and Traction Stresses of Primary Human Macrophages. *Cellular and*
541 *Molecular Bioengineering*. 2016;9(3):455-65.

542 36. Smith LA, Aranda-Espinoza H, Haun JB, Dembo M, Hammer DA. Neutrophil Traction
543 Stresses are Concentrated in the Uropod during Migration. *Biophys J*. 2007;92(7):L58-L60.

544 37. Jannat Risat A, Dembo M, Hammer Daniel A. Traction Forces of Neutrophils Migrating
545 on Compliant Substrates. *Biophys J*. 2011;101(3):575-84.

546 38. Gupta M, Kocgozlu L, Sarangi BR, Margadant F, Ashraf M, Ladoux B. Chapter 16 -
547 Micropillar substrates: A tool for studying cell mechanobiology. In: Paluch EK, editor.
548 *Methods in Cell Biology*. 125: Academic Press; 2015. p. 289-308.

549 39. Ricart Brendon G, Yang Michael T, Hunter Christopher A, Chen Christopher S,
550 Hammer Daniel A. Measuring Traction Forces of Motile Dendritic Cells on Micropost Arrays.
551 *Biophys J*. 2011;101(11):2620-8.

552 40. Cougoule C, Van Goethem E, Le Cabec V, Lafouresse F, Dupré L, Mehraj V, et al. Blood
553 leukocytes and macrophages of various phenotypes have distinct abilities to form
554 podosomes and to migrate in 3D environments. *European Journal of Cell Biology*.
555 2012;91(11):938-49.

556 41. Buccione R, Orth JD, McNiven MA. Foot and mouth: podosomes, invadopodia and
557 circular dorsal ruffles. *Nat Rev Mol Cell Biol*. 2004;5(8):647-57.

558 42. Dufrene YF, Ando T, Garcia R, Alsteens D, Martinez-Martin D, Engel A, et al. Imaging
559 modes of atomic force microscopy for application in molecular and cell biology. *Nat Nano*.
560 2017;12(4):295-307.

- 561 43. Labernadie A, Thibault C, Vieu C, Maridonneau-Parini I, Charrière GM. Dynamics of
562 podosome stiffness revealed by atomic force microscopy. *Proc Natl Acad Sci U S A*.
563 2010;107(49):21016.
- 564 44. Labernadie A, Bouissou A, Delobelle P, Balor S, Voituriez R, Proag A, et al. Protrusion
565 force microscopy reveals oscillatory force generation and mechanosensing activity of human
566 macrophage podosomes. 2014;5:5343.
- 567 45. Kress H, Stelzer EHK, Griffiths G, Rohrbach A. Control of relative radiation pressure in
568 optical traps: Application to phagocytic membrane binding studies. *Phys Rev E: Stat,*
569 *Nonlinear, Soft Matter Phys.* 2005;71(6):061927.
- 570 46. Svitkina TM, Bulanova EA, Chaga OY, Vignjevic DM, Kojima S-i, Vasiliev JM, et al.
571 Mechanism of filopodia initiation by reorganization of a dendritic network. *The Journal of*
572 *Cell Biology.* 2003;160(3):409.
- 573 47. Cojoc D, Difato F, Ferrari E, Shahapure RB, Laishram J, Righi M, et al. Properties of the
574 Force Exerted by Filopodia and Lamellipodia and the Involvement of Cytoskeletal
575 Components. *PLoS One.* 2007;2(10).
- 576 48. Vonna L, Wiedemann A, Aepfelbacher M, Sackmann E. Micromechanics of filopodia
577 mediated capture of pathogens by macrophages. *Eur Biophys J.* 2007;36(2):145-51.
- 578 49. Kress H, Stelzer EHK, Holzer D, Buss F, Griffiths G, Rohrbach A. Filopodia act as
579 phagocytic tentacles and pull with discrete steps and a load-dependent velocity. *Proc Natl*
580 *Acad Sci U S A.* 2007;104(28):11633-8.
- 581 50. Ostrowski Philip P, Grinstein S, Freeman Spencer A. Diffusion Barriers, Mechanical
582 Forces, and the Biophysics of Phagocytosis. *Developmental Cell.* 2016;38(2):135-46.
- 583 51. Freeman SA, Grinstein S. Phagocytosis: receptors, signal integration, and the
584 cytoskeleton. *Immunological Reviews.* 2014;262(1):193-215.
- 585 52. Holevinski KO, Nelson DJ. Membrane capacitance changes associated with particle
586 uptake during phagocytosis in macrophages. *Biophys J.* 1998;75.
- 587 53. Bajno L, Peng X-R, Schreiber AD, Moore H-P, Trimble WS, Grinstein S. Focal Exocytosis
588 of Vamp3-Containing Vesicles at Sites of Phagosome Formation. *The Journal of Cell Biology.*
589 2000;149(3):697.
- 590 54. Braun V, Fraisier V, Raposo G, Hurbain I, Sibarita J-B, Chavrier P, et al. TI-
591 VAMP/VAMP7 is required for optimal phagocytosis of opsonised particles in macrophages.
592 *The EMBO Journal.* 2004;23(21):4166-76.
- 593 55. Herant M, Heinrich V, Dembo M. Mechanics of neutrophil phagocytosis: behavior of
594 the cortical tension. *Journal of Cell Science.* 2005;118(9):1789.
- 595 56. Marion S, Mazzolini J, Herit F, Bourdoncle P, Kambou-Pene N, Hailfinger S, et al. The
596 NF- κ B Signaling Protein Bcl10 Regulates Actin Dynamics by Controlling AP1 and OCRL-
597 Bearing Vesicles. *Developmental Cell.* 2012;23(5):954-67.
- 598 57. Levin R, Grinstein S, Canton J. The life cycle of phagosomes: formation, maturation,
599 and resolution. *Immunological Reviews.* 2016;273(1):156-79.
- 600 58. Evans E, Leung A, Zhelev D. Synchrony of cell spreading and contraction force as
601 phagocytes engulf large pathogens. *The Journal of Cell Biology.* 1993;122(6):1295.
- 602 59. Araki N, Hatae T, Furukawa A, Swanson JA. Phosphoinositide-3-kinase-independent
603 contractile activities associated with Fcy-receptor-mediated phagocytosis and
604 macropinocytosis in macrophages. *Journal of Cell Science.* 2002;116(2):247.
- 605 60. Swanson JA, Johnson MT, Beningo K, Post P, Mooseker M, Araki N. A contractile
606 activity that closes phagosomes in macrophages. *Journal of Cell Science.* 1999;112(3):307.

607 61. Diakonova M, Bokoch G, Swanson JA. Dynamics of Cytoskeletal Proteins during Fcγ
608 Receptor-mediated Phagocytosis in Macrophages. *Molecular Biology of the Cell*.
609 2002;13(2):402-11.

610 62. Boulais J, Trost M, Landry C, Dieckmann R, Levy E, Soldati T, et al. Evolutionary
611 adaptation of phagocytosis modeled the adaptive immune system. *Immunity* 2010.

612 63. Gopaldass N, Patel D, Kratzke R, Dieckmann R, Hausherr S, Hagedorn M, et al.
613 Dynamin A, Myosin IB and Abp1 couple phagosome maturation to F-actin binding. *Traffic*.
614 2012;13(1):120-30.

615 64. Barger SR, Reilly NS, Shutova MS, Li Q, Maiuri P, Heddleston JM, et al. Membrane-
616 cytoskeletal crosstalk mediated by myosin-I regulates adhesion turnover during
617 phagocytosis. *Nature Communications*. 2019;10(1):1249.

618 65. Olazabal IM, Caron E, May RC, Schilling K, Knecht DA, Machesky LM. Rho-Kinase and
619 Myosin-II Control Phagocytic Cup Formation during CR, but Not FcγR, Phagocytosis. *Current*
620 *Biology*. 2002;12(16):1413-8.

621 66. Herant M, Heinrich V, Dembo M. Mechanics of neutrophil phagocytosis: experiments
622 and quantitative models. *Journal of Cell Science*. 2006;119(9):1903.

623 67. Zhelev DV, Needham D, Hochmuth RM. Role of the membrane cortex in neutrophil
624 deformation in small pipets. *Biophys J*. 1994;67.

625 68. Herant M, Lee C-Y, Dembo M, Heinrich V. Protrusive Push versus Enveloping
626 Embrace: Computational Model of Phagocytosis Predicts Key Regulatory Role of Cytoskeletal
627 Membrane Anchors. *PLoS Computational Biology*. 2011;7(1):e1001068.

628 69. Allen LA, Aderem A. Molecular definition of distinct cytoskeletal structures involved
629 in complement- and Fc receptor-mediated phagocytosis in macrophages. *J Exp Med*.
630 1996;184.

631 70. Masters TA, Pontes B, Viasnoff V, Li Y, Gauthier NC. Plasma membrane tension
632 orchestrates membrane trafficking, cytoskeletal remodeling, and biochemical signaling
633 during phagocytosis. *Proc Natl Acad Sci U S A*. 2013;110(29):11875-80.

634 71. Marie-Anaïs F, Mazzolini J, Herit F, Niedergang F. Dynamin-Actin Cross Talk
635 Contributes to Phagosome Formation and Closure. *Traffic*. 2016;17(5):487-99.

636 72. Guevorkian K, Maître JL. Chapter 10 - Micropipette aspiration: A unique tool for
637 exploring cell and tissue mechanics in vivo. In: Lecuit T, editor. *Methods in Cell Biology*. 139:
638 Academic Press; 2017. p. 187-201.

639 73. Herant M, Marganski WA, Dembo M. The Mechanics of Neutrophils: Synthetic
640 Modeling of Three Experiments. *Biophys J*. 2003;84(5):3389-413.

641 74. Vorselen D, Wang Y, de Jesus MM, Shah PK, Footer MJ, Huse M, et al. Superresolved
642 microparticle traction force microscopy reveals subcellular force patterns in immune cell-
643 target interactions. *bioRxiv*. 2019:431221.

644 75. Vorselen D, Wang Y, Footer MJ, Cai W, Theriot JA. Superresolved and reference-free
645 microparticle traction force microscopy (MP-TFM) reveals the complexity of the mechanical
646 interaction in phagocytosis. *bioRxiv*. 2018:431221.

647