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Ca2 + dynamics correlates with phenotype and function in primary human neutrophils

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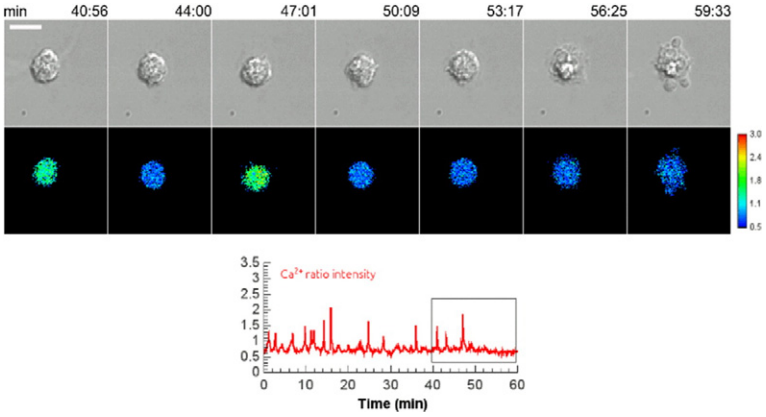
HIGHLIGHTS

- Multivariate live-cell imaging of different functional states of human primary neutrophils.
- First live-cell measurements of long sustained calcium oscillations.
- Different calcium dynamics correlates.

GRAPHICAL ABSTRACT



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ABSTRACT

Central to the immune defense function of neutrophils is to sense, to move and to kill. Neutrophils acquire distinct cellular states necessary to fulfill these functions each associated with a particular phenotype. The cells constituting the neutrophil population are presumably not synchronized with respect to their actual state, e.g. due to maturity or preactivation. It is also likely that they exhibit a different degree of phenotypic plasticity (that is, the ability to switch to a particular state). Calcium is known to play a crucial role in neutrophils such as for cell motility. The present study focuses on characterizing the cell-to-cell variability at the morphological as well as at the level of calcium dynamics by studying single primary human neutrophils. We apply long-term multivariate live cell imaging to (i) characterize neutrophil phenotypes of different functional states, (ii) analyze the distribution of cells being in these states and, (iii) study the individual intracellular calcium response simultaneously with shape changes. We are able to differentiate the five distinct subpopulations of neutrophils based on quantitative parameters of cell morphology and motility. As a major result, we demonstrate that the calcium dynamics of individual cells correlates with their respective functional state. Finally, we see a number of cells that undergo spontaneous phenotypic changes from one cellular state to another. These events are preceded either by exhibiting the calcium dynamics of the future state or by switching to the respective calcium dynamics in parallel to switching the morphology. Based on our results we conclude that specific calcium dynamics carries crucial information for the function and phenotype of neutrophils.

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1. Introduction

The efficiency of our immune response critically depends on the ability of neutrophils which represent the major type of white blood cells in mammals to sense inflammation-related signals, move to sites of infections or tissue injury and initiate killing or repair mechanisms [1]. Impairment of these processes on one hand leads to life-threatening infections as with the leukocyte adhesion deficiency syndrome or with the chronic granulomatous disease [1]. On the other hand, hyperactivation or the failure to inactivate neutrophils could participate in perilous chronic inflammation and tissue damage [2]. Therefore, deepening our understanding of the mechanisms underlying the activation of neutrophils may offer new ways to deliberately restore or shut-down neutrophil function which is of major clinical importance. Upon activation neutrophils pass through distinct cellular states, each associated with a particular phenotype and activity, e.g. adhesion, migration or phagocytosis [3]. The biochemical processes involved are diverse and most probably differently activated and intertwined in the different states. Finally, many of the neutrophil functions require substantial changes in the cell shape which are also state-specific. Despite extensive studies, it remains unclear, how neutrophils integrate the multitude of biochemical and mechanical signals.

There are only few attempts to phenotypically define different subsets of neutrophils [4,5]. Keller and coworkers published first attempts to differentiate between phenotypes by reflection-contrast microscopical pictures and parameters like the cell length or area of contact with the substratum [6]. In addition, they report differences in response to fMLP (Formyl-Methionyl-Leucyl-Phenylalanine), a known agonist. Thus, they describe spherical (which we call round in the following), spread and motile (with protrusions or crawling) cells. They also mention the observation of blebbing cells and cells that undergo changes from one phenotypic class to another. However, the authors' intention is not to use quantitative parameters for the semi-automated classification of different subclasses and therefore, there is only limited quantitative information which could be used for this purpose. In any case, using the contact area does not allow the differentiation between cells showing protrusions compared to crawling cells, since these have the same contact area. In addition, blebbing cells are not described in this study.

It is not very well understood, how phenotypic plasticity, i.e. the cell-fate decision is regulated, either to remain in or to switch to another particular state. Since all the cells constituting the neutrophil population are presumably not synchronized with respect to their actual state due to differences in maturity or pre-activation etc., it is not very likely that each cell is capable of adopting any particular state and fulfilling any particular function.

The present study addresses this issue and is intended to shed light into the phenotypical heterogeneity coupled to distinct biochemical states across the neutrophil population. We investigate primary single neutrophils and apply time-resolved multivariate live cell imaging of shape and calcium dynamics to account for cell-to-cell variations. Calcium dynamics as an intracellular process has been chosen in this first attempt as it is known to be a crucial mediator of many of the neutrophil functions [7]. Thus, we followed the notion that, different morphological states that are likely representing different functional states, are also accompanied by distinct calcium dynamics. If this is the case, calcium dynamics can be used as an additional classifier for the differentiation of different subpopulations and of course reflects different signaling states of the cell. Even though the involvement of calcium in diverse processes is known, it is not at all obvious, if the calcium dynamics – observed as an integrated signal over the cell – will be distinct between the different states.

On the molecular level, it is known that intracellular calcium is among many other functions an essential modulator of all stages of the cell migration cycle [8]. In neutrophils, calcium concentration rises following adherence and activation via integrin and chemotactic receptor signaling, respectively [9]. Calcium influx at the plasma membrane has been shown to integrate chemotactic and adhesive signals, and functions to synchronize signaling of neutrophil arrest and migration [10]. Furthermore, vesicular integrin recycling to the leading edge of a migrating neutrophil is facilitated by calcium [11]. Calcium modulates cytoskeletal rearrangements by regulating the activity of actin binding proteins (ABP) such as gelsolin mainly leading to disassembly of actin networks. Moreover, calcium regulates traction formation, adhesion disassembly and retraction by being a main activator of the myosin light chain kinase (MLCK). It was demonstrated to be required for uropod retraction during neutrophil migration through myosin II activation [12]. Besides its role in controlling neutrophil chemotaxis calcium contributes to other neutrophil functions such as the production of reactive oxygen species through activation of the NADPH oxidase via Ca-dependent protein kinase C [13] or in promoting degranulation [14–16]. In summary, there is a wealth of processes which involve calcium signaling and it is clear that different processes will be of different importance at different functional states.

In addition to previous studies describing different functionalities of neutrophils based on solely inspecting morphology — as described above [5,6], some studies attempted to depict differences by describing solely the calcium dynamics (to name only a few [17–20]) or to a lesser extent the correlation of calcium and morphology [21–24]. Most of the studies address a particular functional activity (i.e. a particular subgroup of the neutrophil population) and describe observations during a rather short period of time such as during the initial onset of a calcium transient or the respective motility response. Thus, calcium transients have been studied during initial adherence [17,22], chemokinesis [19], chemotaxis [24–26] and phagocytosis [22,25,27–30] both in the presence and absence of chemotactic stimuli.

In this study we aim at revealing simultaneous and spontaneous behaviors of cells constituting the neutrophil population followed during long periods of time. Our results demonstrate the coexistence of certain subpopulations of cells for which we present a detailed quantitative classification scheme taking into account multiple parameters obtained from cell morphology. These parameters were based on a classification scheme for lymphocytes published by Eisele et al. [31]. We furthermore observe that the proportion of subpopulations can be altered depending on the microenvironment. In addition, we show that cells which represent a certain functional state do not only share morphological characteristics but also commonly exhibit the same qualitative calcium dynamics. This is also evident during spontaneous state transitions. Due to our comparatively long measurements (up to 60 min), we are able to observe a number of such transitions. It appears that the calcium dynamics exhibited by the cells undergoing transitions either changes in parallel or – as observed in several cases – already changed prior to the morphological characteristics. This is one of few cases where a particular macroscopic function clearly correlates with specific calcium dynamics. Previously reported correlations mostly focus on gene expression following the seminal work by Dolmetsch et al. [32] and Li et al. [33].

2. Results

2.1. Morphological characterization of neutrophil phenotypes

We first inspect the cell-to-cell variations at the morphological level in terms of the three criteria (i) shape (morphological appearance), (ii) motility (shape changes in time), and (iii) movement (cell net displacement in time and space) using time-resolved live cell differential interference contrast (DIC) images. In order to quantitatively describe this we use a combination of different morphological parameters, such as the area, extension, dispersion and locomotion (see the Materials and methods section). Based on these we identify five distinct subpopulations which we name according to certain morphological characteristics as: round, spread, crawling, agile, and blebbing. These are in turn associated with distinct functional properties. 1.

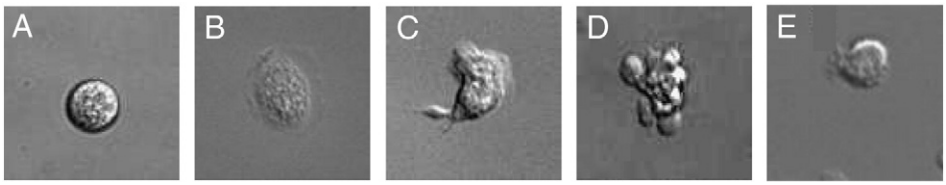

Fig. 1. Characteristic morphological appearance of neutrophil functional states imaged by DIC. Round (A), spread (B), crawling (C), blebbing (D) and agile (E).

Round cells are of spherical shape (small extension and dispersion) with a very small area. They neither show substantial changes in these shape parameters in time nor locomotion (Figs. 1A and 2). 2. Spread cells exhibit a large area as they acquire a flattened morphology on the substratum and may show membrane ruffling at the cell periphery (low mean dispersion and extension). Spread cells are tightly adherent, and do not show significant locomotion (Figs. 1B and 2). 3. Crawling cells undergo shape changes known to occur during cell migration, i.e. polarization, formation of lamellipodia at the front and retraction of the uropod at the rear end. Specifically, only for crawling cells extensive shape deformations (i.e. changes in area, dispersion and extension) are accompanied by substantial cell locomotion (Figs. 1C and 2). 4. and 5. Both, agile and blebbing

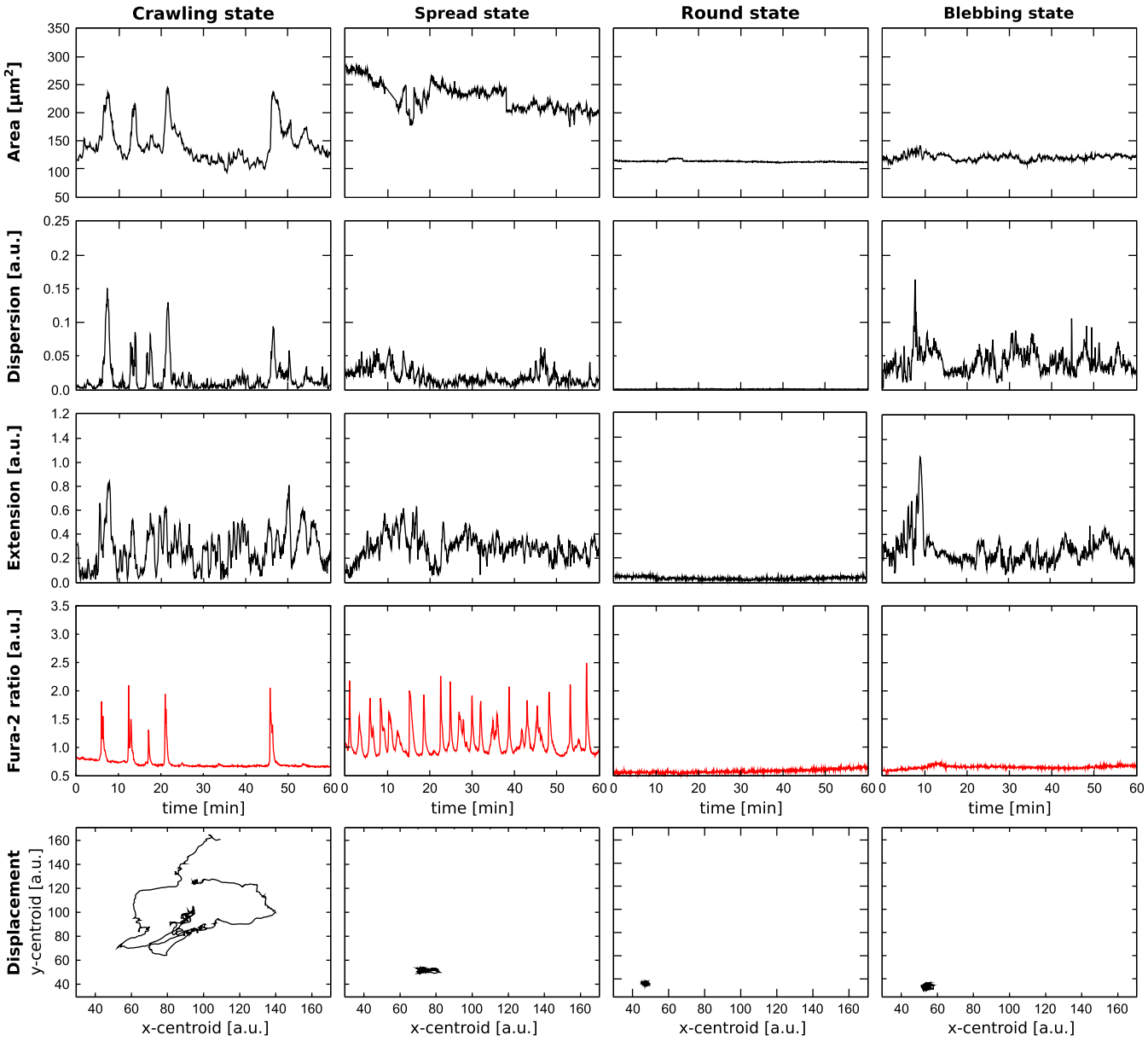

Fig. 2. Morphological parameters and calcium dynamics of different phenotypes. *Top three rows*: State characteristic time series of shape parameter values given in absolute quantities *Fourth row*: State characteristic time series of intracellular calcium dynamics given in ratio intensity of Fura-2 (red). *Fifth row*: Locomotion given as the change in the x–y-centroid (black). Since agile cells show a more variable picture w.r.t. calcium dynamics (see Table 1), no single typical cell is displayed here.

cells (Figs. 1D,E and 2) display rather constant area and small locomotion but posses large changes in extension and dispersion. This allows us to distinguish them from the other subpopulations but, however, not between them. Discrimination based on just morphology is currently only possible by visual inspection. Agile cells show spike-like protrusions in the form of thin filipodia in contrast to the spherical membrane protrusions of blebbing cells (Fig. 1D and E). The typical morphological appearance of different neutrophil subpopulations as well as corresponding representative time evolutions of the morphological parameters of about 60 min is displayed in Figs. 1 and 2, respectively. In addition, a statistical analysis of all cells with respect to the morphological parameters is displayed in Fig. 3. Thus, using pair-wise t-tests on the populations and in accordance to the above data, locomotion as a measure allows the separation crawling cells from all others, whereas populations of spread cells can be identified by their area. Round cells are identifiable by their extremely low dispersion and locomotion. Finally, agile and blebbing cells have larger dispersion than the others and still differ in dispersion from each other. However, given the fact that we are interested in single cell analysis, population means are only of limited value. With this in mind, we have to apply measures that distinguish not only means, but also all cells (or close to all cells). Here, locomotion is a certain predictor of crawling cells (all analyzed cells show higher locomotion than all others),

Mean area

	a	b	c	r	s
a	-	1	1	0	1
b		-	0	1	1
c			-	1	1
r				-	1
s					-

Locomotion

	a	b	c	r	s
a	-	0	1	1	0
b		-	1	1	0
c			-	1	1
r				-	1
s					-

Mean dispersion

	a	b	c	r	s
a	-	1	1	1	1
b		-	1	1	1
c			-	1	1
r				-	1
s					-

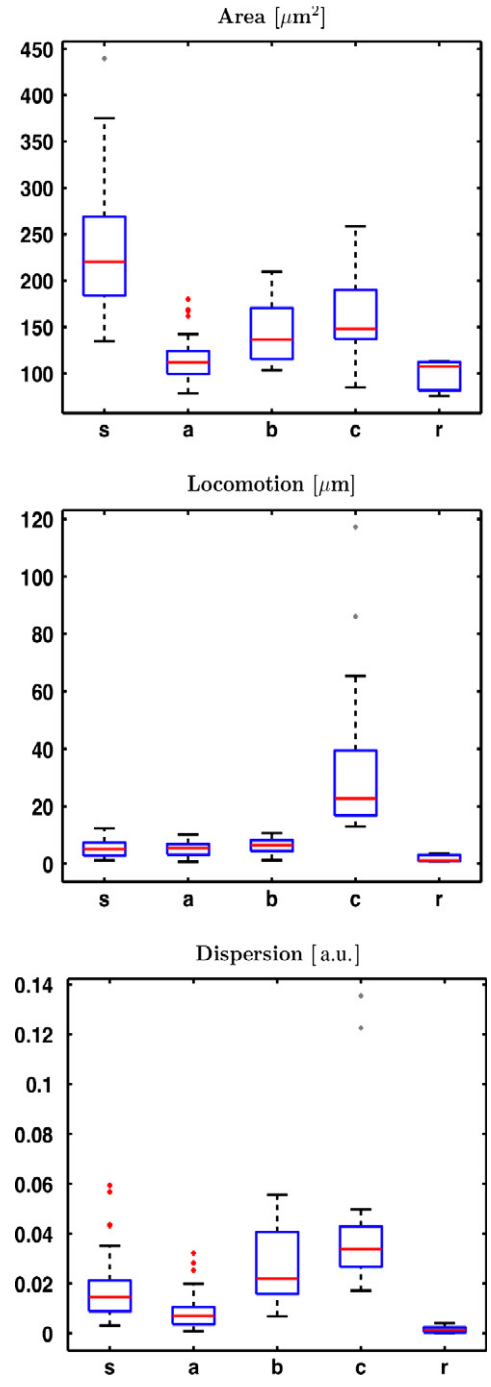

Fig. 3. Statistical analysis of morphological parameters of different phenotypes. Mean area, locomotion and mean dispersion are displayed as box plots for all measured cells of the different phenotypic populations (numbers as in Table 1). In addition, results of a pair-wise *t*-test are shown next to the respective box plots. In the Table 1 stands for a significant difference (confidence value $p < 0.05$) between individual populations, where 'a' represents agile cells, 'b' blebbing cells, 'c' crawling cells, 'r' round cells and 's' spread cells.

and the low dispersion of round cells allows the separation of these completely from the others. For all other purposes, combinations of morphological parameters plus another measure are needed.

2.2. Calcium dynamics of neutrophil subpopulations

Together with the morphological images we simultaneously acquired time-resolved live cell intensities of the ratiometric calcium indicator Fura-2. This way, we monitor changes in the intracellular calcium concentration in single cells. The results (Fig. 2) reveal cell-to-cell variations in the neutrophil population ranging from basal steady-state to one or several single calcium peaks or to sustained complex oscillatory behavior. Here, complex indicates that the calcium oscillations often represent a mix of bursts (peaks followed by secondary oscillations at an elevated state before returning to the basal state) and spikes (no secondary oscillations) and do show variations in amplitude and frequency probably indicating stochasticity. For the current study and for simplicity reasons, we ignored these variations and – in accordance to these observations – we defined three different categories of calcium dynamics, i.e. basal (steady state), occasional spikes (with periods of no calcium activity of at least 4 min occurring several times and consecutively) and sustained calcium oscillations, respectively (Fig. 2). Interestingly, we find a strong correlation between the calcium dynamics of a cell and its functional phenotype. In other words, cells that belong to a certain subpopulation with respect to DIC parameters also exhibit reproducibly similar calcium dynamics. Thus, for each individual cell we analyzed its respective morphological and calcium dynamics characteristics. This is summarized in Table 1. To our knowledge, we present for the first time sustained long-term complex calcium oscillations in neutrophils, namely in spread cells. 23 out of 30 cells exhibit these sustained oscillations whereas the rest exhibits occasional calcium spikes and none of the spread cells showed only a basal calcium level. In contrast, 9 out of 12 crawling cells only showed occasional spikes. Most notably, we observe that every single calcium peak of these crawling cells coincides with a single large locomotion event and is accompanied with substantial changes in the cell's area and dispersion (Fig. 4). However, 3 of the analyzed crawling cells also showed sustained calcium oscillations; none showed an only basal calcium level. A very different picture results from the analysis of round and bleb-forming cells: In contrast to the other states, only steady-state levels of calcium can be detected here. A more complex picture arises from the analysis of agile cells. Here, we also observe occasional single calcium spikes in more than half the cells which are often accompanied by protrusion formation, e.g. when the cell appears to start migrating but fails because it remains anchored to the surface. However, protrusion formation is sometimes still hard to capture with the existing segmentation algorithms plus there are also cells which do not show any calcium dynamics. In this last case, the automatic classification including the calcium transients would still not allow the differentiation of agile and bleb-forming cells whereas the cells exhibiting occasional calcium peaks could be detected as agile by the automated analysis.

2.3. Neutrophil plasticity

Obviously, the term “distinct subpopulation” does not imply that each cell belongs to the same subpopulation all the time. In fact, we observe one third of cells changing their state which we call transient cells (Table 2). However, the heterogeneity of the cells will also be reflected in differences in their respective ability to switch from one specific state to another. This might be also influenced by the environment to which the cells are subjected.

To study the impact of environmental conditions on the proportion of the neutrophil subpopulations we used two different conditions — one more physiological and one that is supposed to improve the motility of the cells (containing BSA, see the Materials and methods section). We use these two standard buffers as a simple means to check whether the proportion of subpopulations change and whether our observations w.r.t. the correlation of morphology and calcium dynamics hold under different conditions rather than studying the particular influence of one specific chemical species in the buffer. Using the buffer which is supposed to improve motility, we indeed observe reduced adhesiveness of cells and an altered distribution of the neutrophil subpopulations in the latter environment (Table 2). Thus, the motility increasing medium substantially reduces the number of observed spread cells. In contrast, agile and crawling cells are more abundant. Again, the morphological phenotype correlates with the respective calcium dynamics.

Analyzing the cells undergoing state transitions with respect to their calcium dynamics reveals that a change in morphological parameters is accompanied by a change in calcium dynamics such that the above observed correlation between the two is also visible during transitions. Interestingly, cells also start to exhibit the specific calcium dynamics of the new state often some time before the actual morphological parameters change. Thus, the most common transitions observed were transitions from agile to bleb-forming (12) and from spread to bleb-forming (12). In 9 out of the 12 cases each, the respective cells already showed only basal calcium levels during the observation period before they actually changed their morphological parameters. In 3 cases each, occasional spikes (agile cells) or calcium oscillations (spread cells) stopped in parallel to the change of morphological parameters. As pointed out in more detail in the Discussion section, this could be indicative of a transition to apoptosis. In addition to these transients, we observed single cells with different diverse transitions. In almost all cases, the calcium dynamics coincides before and after the change with the above observed correlation. However, since single observations are obviously not statistically significant we do not describe them in detail here. Fig. 5 shows an example of an agile cell switching into a blebbing cell, whereas Fig. 6 represents an example of a cell changing from the spread state to the blebbing state.

Table 1
Calcium dynamics of non-transient cells.

n = 70	1 (Basal steady state) n (%)	2 (Cont. oscillations) n (%)	3 (Occasional spikes) n (%)
Agile	7 (37)	1 (5)	11 (58)
Blebbing	5 (100)	–	–
Crawling	–	3 (25)	9 (75)
Round	4 (100)	–	–
Spread	–	23 (77)	7 (23)

3. Discussion

We have investigated primary human neutrophils on a single cell level in order to shed light into the functional and biochemical heterogeneities across the neutrophil population. For this purpose, multivariate quantitative data were obtained from live cell imaging of cell morphology and intracellular $Ca2+$ changes over periods of up to 60 min and in different microenvironments. We were able to characterize the known functional states of neutrophils – round, agile, spread, crawling and blebbing – by visual inspection as well as by using the time-resolved quantitative morphological parameters area, extension, dispersion and locomotion. As described above a differentiation between agile and blebbing cells solely on the basis of these parameters is however not possible.

Such a systematic time-resolved assessment has yet not been done for neutrophils. However, the validity of using multiple shape parameters (such as area, extension, dispersion) to discriminate between different subpopulations of white blood cells has been shown for fixed lymphocytes [31].

In addition, we have demonstrated that specific types of calcium dynamics correlate with the phenotype and function of neutrophil subpopulations. Most strikingly, we observed long-term sustained complex calcium oscillations in adherent spread cells, a correlation of peaks and

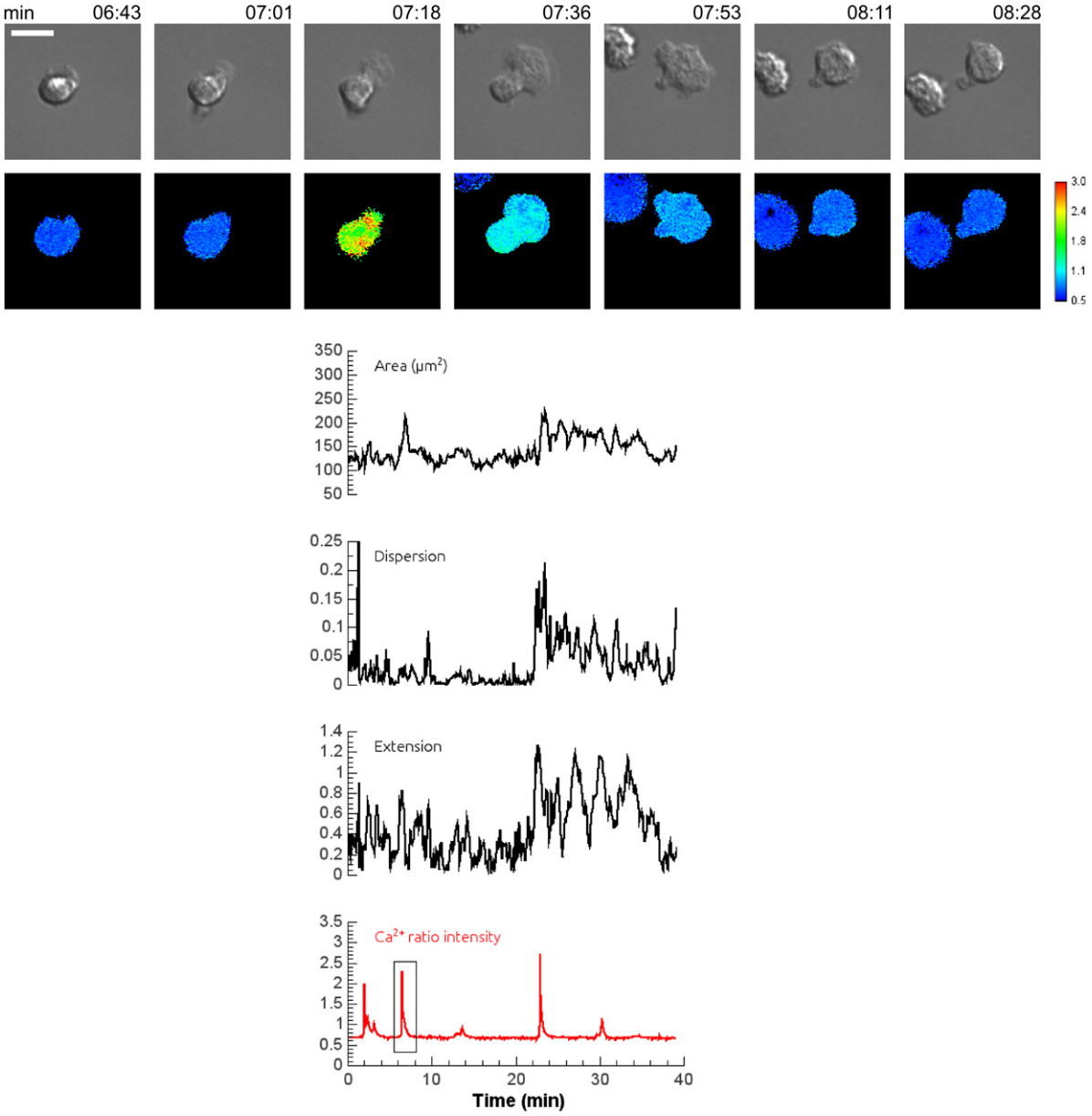

Fig. 4. Quantitative characterization of a crawling cell. Time (min) lapse images around a particular locomotion event (see black box in the calcium dynamics graph). First: DIC images. Second: Ratiometric fluorescent images. Third: Correlation of intracellular calcium dynamics with shape changes, i.e. area, dispersion, and extension. Scale bar: 10 µm. Experimental condition: Buffer B (no BSA).

motility events in crawling cells and occasional spontaneous single calcium spikes in the majority of agile cells. In contrast, round as well as blebbing cells showed rather inconspicuous calcium dynamics. The former is in agreement to what has been reported by [19,21]. To the best of our knowledge, no long-term calcium oscillations in neutrophils have been published. In addition, no calcium transients in agile or blebbing neutrophils – short or long-term – have been reported before.

Whether sustained calcium oscillations can be spontaneously induced in single adherent neutrophils has been a matter of debate for many years. Despite the pioneering observation by Jaconi et al. (1988) [18], the oscillations in solely polarized spread cells reported by Marks and Maxfield (1990) [19], and the short term assessment of multiple calcium transients of round cells undergoing adherence [21] the majority of studies could not detect sustained spontaneous Ca2 + spiking in single adherent human neutrophils [20]. Interestingly, the pattern of the complex calcium oscillations we have observed in spread neutrophils is very similar to those reported for spread mouse macrophages [34]. In accordance to other studies we see that initiation of spreading (quantified by e.g. an increase in the cell area) always follows an increase in calcium [17,22,24]. The strong variation of amplitude and

Table 2
Frequency of occurrence of cellular states in neutrophil subpopulations.

	Total	Medium A[a]	Medium B[b]
	n (%)	n (%)	n (%)
Cells total	104	50	54
Non-transient	70 (66.7)	35 (66.7)	35 (64.8)
Transient	34 (33.3)	15 (33.3)	19 (35.2)
Number of observed states	143	65	78
Agile	40 (28.0)	31 (47.7)	9 (11.5)
Blebbing	30 (21.0)	14 (21.5)	16 (20.5)
Crawling	16 (11.2)	14 (21.5)	2 (2.6)
Round	5 (3.5)	2 (3.1)	3 (3.9)
Spread	52 (36.4)	4 (6.2)	48 (61.5)

[a] 0.5% BSA, 12 mM glucose.
[b] No BSA, 5 mM glucose.

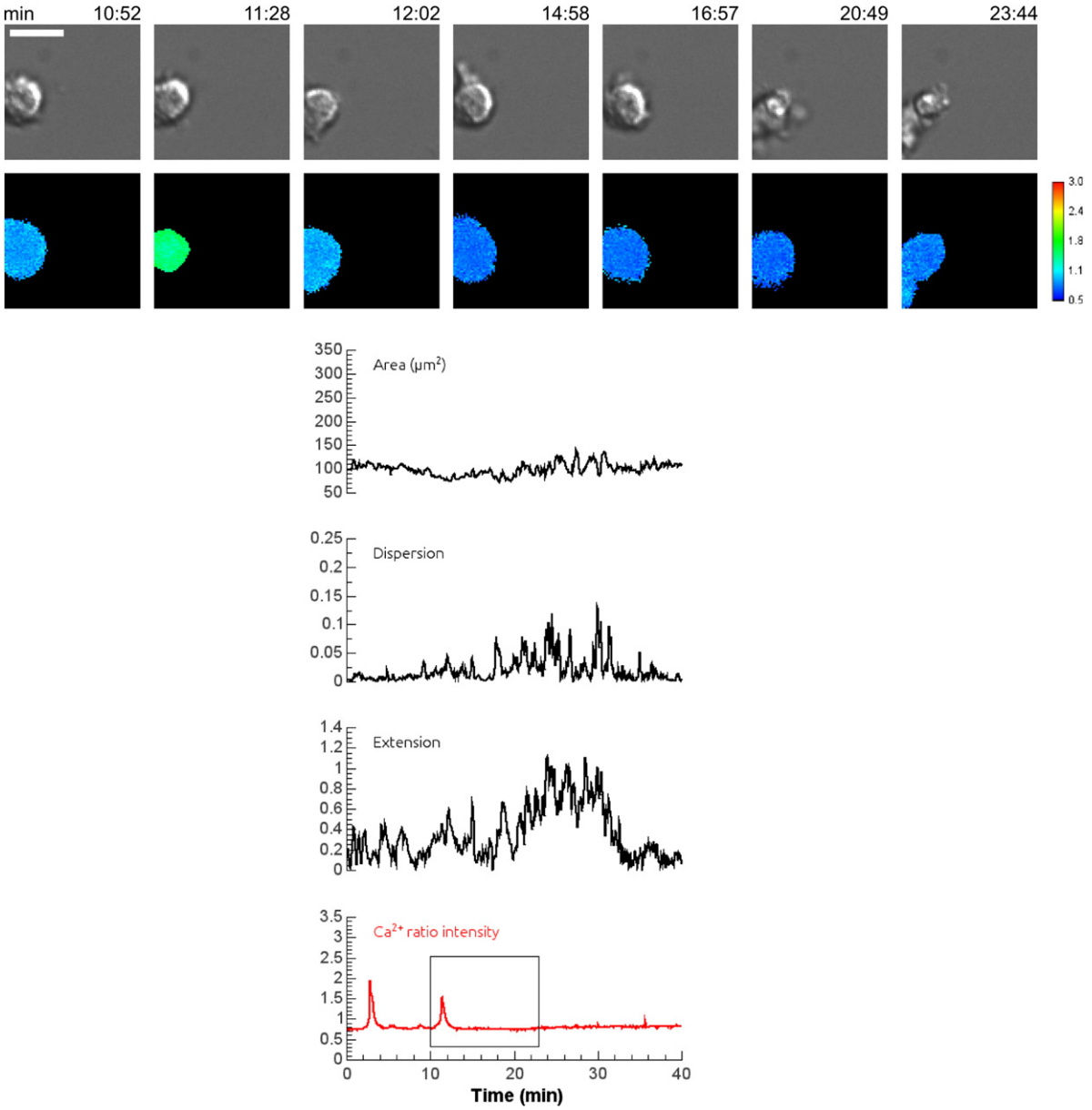

Fig. 5. Quantitative characterization of a state transition from agile to blebbing. Time (min) lapse images around a particular state transition (see black box in the calcium dynamics graph). First: DIC images. Second: Ratiometric fluorescent images. Third: Correlation of intracellular calcium dynamics with shape changes, i.e. area, dispersion, and extension. Scale bar: 10 µm. Experimental condition: Buffer A (0.5% BSA).

frequency, as well as the shape of the calcium oscillations indicates that these are influenced by stochasticity, similar to many other cell types [35]. The need for a stochastic description when quantitatively modeling observed calcium data has been already discussed in 1993 [36], reviewed in [35] and appropriate methods to analyze the experimental data accordingly have been published and are available [37]. Therefore, we will have to take this aspect into account when quantitatively describing the observed dynamics in the future.

Calcium waves in single cells as reported by Kindzelskii and Petty [38] were shown to not be reproducible later on [39] and are not discussed here. Similarly, it is still an unresolved question if Ca2 + transients are a prerequisite for crawling movements [19,23,26]. Our data clearly indicate that calcium peaks of randomly migrating cells coincide reproducibly with single locomotion events. Here, shape and calcium dynamics appear to be temporally interrelated such that an increase in the calcium level may follow a change in the dispersion due to the polarization of the cell but precedes the change in the cell area due to lamellipodium formation before initiation of cellular movement.

We have reported that one third of the cells investigated were subject to phenotypic changes. Notably, when cells switched from one functional activity to another they also switched to the respective state-specific calcium dynamics or already displayed the calcium dynamics typical for the future state well in advance to the actual morphological state. This is especially holds true for the onset of blebbing which we observed for sufficient times to state that this is reproducible and significant. In contrast to crawling and spread neutrophils, there is very little literature on the blebbing state of neutrophils. In general, the view that blebbing represents a hallmark of apoptosis has been challenged by more recent studies suggesting that blebbing may be an important motility mechanism of cells migrating in 3D environments alternatively to the lamellipodia-driven movement [40]. In our study, the important observations with respect to blebbing cells

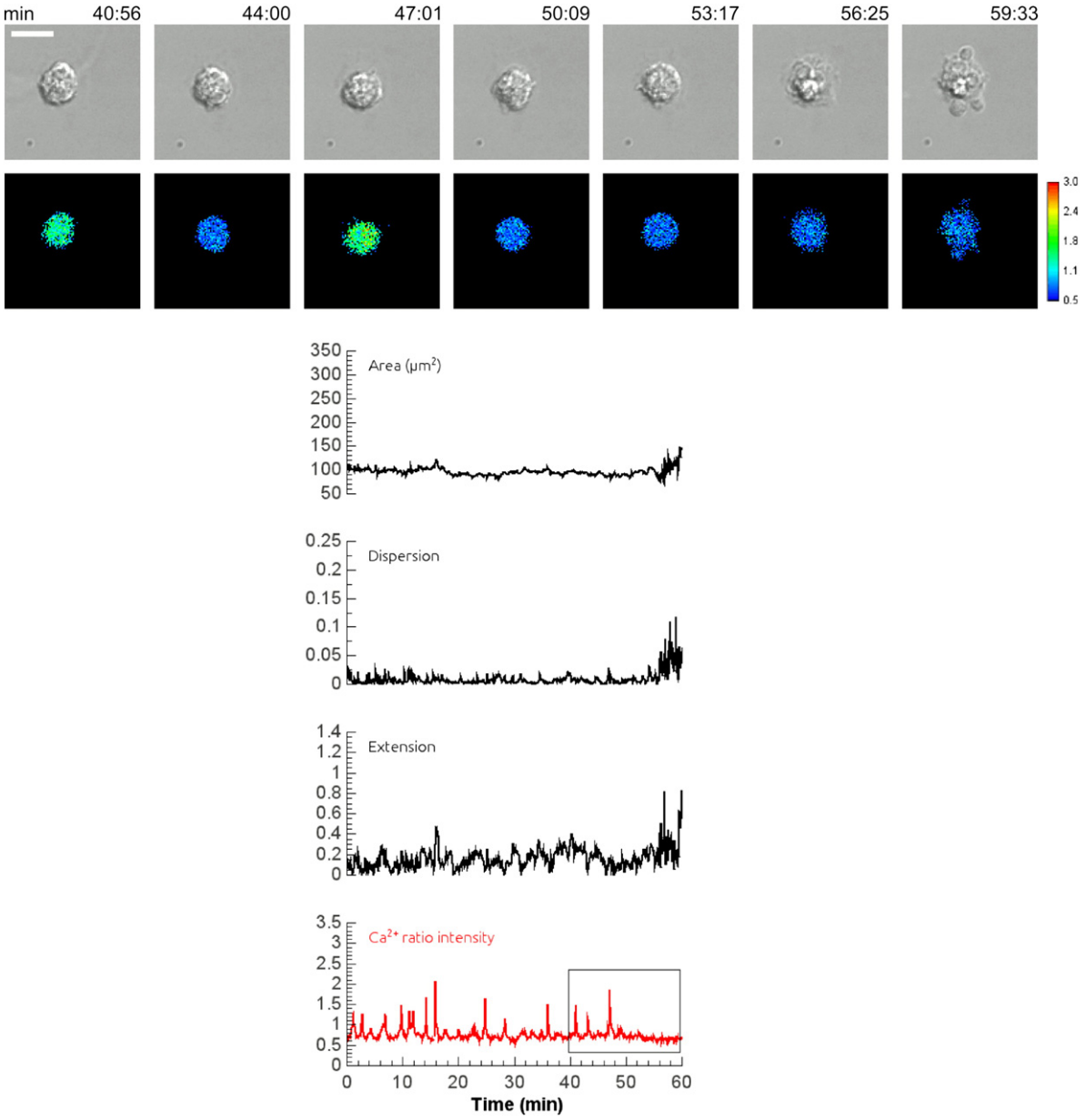

Fig. 6. Quantitative characterization of a state transition from spread to blebbing. Time (min) lapse images around a particular state transition (see black boxes in the calcium dynamics graph). First: DIC images. Second: Ratiometric fluorescent images. Third: Correlation of intracellular calcium dynamics with shape changes, i.e. area, dispersion, and extension. Scale bar: 10 μm. Experimental condition: Buffer B (no BSA).

are that: (i) initiation of bleb formation is preceded or paralleled by the complete halt of any significant calcium dynamics and (ii) in a number of cases blebbing is seemingly associated with persistently increased levels of calcium. The fact that this seems to be irreversible and in contrast to other state transitions, we did not observe a single transition from blebbing to a different state, speaks were in favor of the hypothesis that blebbing in neutrophils (at least at these conditions) precedes apoptosis.

The molecular mechanism underlying the tight interplay between calcium dynamics and morphological changes in neutrophils might lie in the β2 integrin-mediated mechanism of calcium signaling. Thus, the strength of adhesiveness, i.e. the number of activated integrin receptors as also proposed by others [17,21,41] will initiate different calcium signaling states which in turn leads to different changes in the morphology. Thus, the tightly adherent spread cells occupying a large area presumably exhibit a comparably large number of established adhesive contacts to the substratum which could be a sufficient stimulus to allow for sustained calcium oscillations. These calcium oscillations potentially might then stabilize this functional state.

Finally, we demonstrated that the proportion of the different subpopulations and the accessibility to phenotypic changes vary depending on environmental conditions. Notably, a medium supposed to improve the motility of cells (in this case containing BSA) reduced the adhesiveness of neutrophils to the substratum in line with previous data by others [7]. We also observe an altered distribution in the subpopulations, i.e. an abundance of agile and crawling cells.

The present study shows the usefulness of multivariate live cell imaging for studying cell-to-cell variations across the human neutrophil population on a single cell level. The approach taken provides valuable insight into the morphological as well as the biochemical differences of certain neutrophil subpopulations. Future analyses are expected to shed light into prerequisite conditions necessary to induce specific neutrophil behavior in order to overcome impaired neutrophil functions in the course of certain disease environments.

So far, only few studies, e.g. on the fertilization [42] or neuronal development [43] show a clear correlation between macroscopic cellular function and calcium dynamics. Therefore, our results are important in a more general way than just in the field of neutrophil cell biology.

4. Materials and methods

4.1. Ethics statement

All human volunteers signed an informed consent form and the procedure was approved by S163/2010 of the "Ethics committee of the University of Heidelberg".

4.2. Materials

All chemicals (if not otherwise stated) were from Sigma-Aldrich. Assay buffer A (Hanks' balanced salt solution (HBSS), 27.5 mM Tris pH 7.3, 1.87 mM CaCl2, 0.8 mM MgSO4) supplemented with 12 mM glucose and 0.5% BSA and assay buffer B (20 mM HEPES, 1.1 mM KH2PO4, 130 mM NaCl, 4.6 mM KCl, 5 mM NaHCO3, 1 mM CaCl2, titrated with NaOH, pH 7.4) (as in [43]) supplemented with 5 mM glucose; the fluorescent ratiometric calcium indicator Fura-2/AM [44] was purchased from Invitrogen; and ibiTreat μ-slides 8-well (tissue culture treated) microscopy chambers for live cell imaging were from Ibidi.

4.3. Neutrophil isolation from human peripheral blood

Blood from volunteers was drawn into heparin-coated tubes (Sarstedt, Nümbrecht, Germany). PMNs were isolated from the heparinized blood by PolymorphPrep™ (Nycomed, Oslo, Norway) followed by hypotonic lysis buffer to remove erythrocytes. Neutrophils were suspended in either buffer A or buffer B at a final concentration of 10^6 cells/ml. Cells were kept on ice until use.

4.4. Loading the cells with Fura-2/AM

1 ml of neutrophil suspension (10^6 cells) was centrifuged for 5 min at 200 *g*. Cell pellets were resuspended in 1 ml of the same buffer (A or B) as they have been in before containing 1 μM Fura-2 as the acetoxymethyl ester (Fura-2/AM). After incubation for 30 min at room temperature cells were centrifuged, washed twice and resuspended in the respective assay buffers.

4.5. Live cell imaging

250 μl of Fura-2 loaded neutrophil suspension was seeded onto ibiTreat μ-slides. Cells were allowed to adhere for about 10–15 min. Cell morphology was monitored by imaging differential interference contrast (DIC). Fura-2 is a fluorescent dual excitation (ex) dye meaning Ca2+ shifts its excitation maximum to shorter wavelengths from 387 nm for Ca-unbound to 340 nm for Ca-bound Fura-2. The ratio of the emissions (em) at those wavelengths (R = F340/F387) is directly correlated to the amount of intracellular calcium. Sequences of widefield image triplets (DIC, Ca-bound and Ca-unbound Fura-2) were acquired for about 1 h on a Nikon Ti inverted microscope equipped with an ORCA-AG CCD camera (Hamamatsu), 40×/1.3 NA oil immersion objective, a perfect focus system (PFS) and NIS Elements AR 3.20 software (all Nikon, Tokyo, Japan). Imaging was performed at the Nikon Imaging Center Heidelberg. Excitation wavelengths were changed by filter switching. Default microscopy settings were: Channel 0 (Ca-bound Fura-2, ex 340 nm, em 510 nm, exposure time 100 ms, gain 5.0, binning 2 × 2), Channel 1 (Ca-unbound Fura-2, ex 387 nm, em 510 nm, exposure time 10 ms, gain 5.0, binning 2 × 2), Channel 2 (DIC, exposure time 50 ms, gain 1.2, binning 2 × 2). The average frame rate was 0.285 fps.

4.6. Image processing and data analysis

In total 104 cells obtained from two individuals and studied in 9 different experiments were analyzed as follows. NIS Elements ND2 files were imported into ImageJ using the LOCI Bio-Formats plugin. Fluorescence intensities of the two Fura-2 channels were background subtracted. Images were then automatically analyzed using the QuimP software version 2 [45], a plugin for ImageJ that enables simultaneous quantification of membrane movement and fluorescence intensity. QuimP consists of two independent modules, BOA and ANA, which provide automatic cell boundary tracking and intensity sampling, respectively. As the basis for BOA we used the DIC images. In case of low contrast we used Find Edges and Gaussian Blur beforehand to improve the cell segmentation performance by QuimP2. In the end, the cell's contour is represented as a chain of connected nodes (outer chain). The QuimP2 ANA plugin was then applied to all three channels. Given a certain value (cortex width) ANA creates a second chain of connected nodes (inner chain) which roughly represents the border between cell cortex and body. ANA generates comprehensive output data such as the tracking number of each node and many other information about the cell, such as x- and y-coordinates of the centroid, cell area, extension and fluorescence intensities (cortex and body). Morphological parameters and ratio intensities were plotted using Octave and Qtiplot.

4.7. Visualization of ratiometric images

To correct for observed border effects where irregularly high or low fluorescence ratios appeared, such as in the border region around cells due to the fluorescent signal in either one of the two channels being only slightly above the background, a manual thresholding procedure was performed. For this, an approximate region of interest (ROI) outside but in close proximity to the cell was selected in the DIC channel and the intensity distribution within this region was assessed in the 387 channel. A cutoff was then determined in a way that it included about 95% of the signal in the ROI. The entire image was then thresholded using the cutoff value and all pixels below the cutoff were set to zero. Ratiometric images were then obtained by dividing the pixel-wise background-corrected intensity in the 340 channel by the corresponding intensity in the 387 channel. For pixels with zero intensity in the 387 channel the ratio was set to zero.

4.8. Characterization of neutrophil subpopulations

The parameters we use to quantitatively characterize the cell's morphology are: x- and y-coordinates of the centroid, cell area, extension (how much the shape of the cell differs from a circle (=0 if the shape is circular)), dispersion (smoothness of the cell contour) as well as locomotion (as the sum of squares of the difference of the maximum and minimum x–y centroid coordinates). The cell trajectory is visualized as the movement of the centroid in time.

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