Sack U, Tárnok A, Rothe G (eds): Cellular Diagnostics. Basics, Methods and Clinical Applications of Flow Cytometry. Basel, Karger, 2009, pp 325–342

Determination of Cell Physiological Parameters: pH, Ca^{2+}, Glutathione, Transmembrane Potential

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Introduction/Background

The determination of physiological properties of cells will be exemplified using phagocytic cells. Professional phagocytes such as neutrophils, eosinophils, basophils and monocytes/macrophages play a pivotal role in the nonspecific or innate immune response. These cells remove cell and tissue debris, apoptotic cells and foreign particles. Furthermore, phagocytes are involved in inflammatory reactions and inhibit tumor growth. Monocytes exert their influence via the expression of tissue factors and their interactions with thrombocytes, homeostasis and wound healing [1]. Phagocytes can engulf microorganisms and thereby induce their destruction. In addition, they can present bacterial antigens via the major histocompatibility (MHC) system and induce a specific adaptive immune response. Via this mechanism, phagocytes link the innate with the adaptive immune response. The cascade of phagocytic responses to microorganisms consists of a complex sequence of graded processes: chemotaxis, actin polymerization, migration, aggregation, phagocytosis, degranulation, release of reactive oxygen species (O_2^-, H_2O_2) and changes in the intracellular pH (pH_i), cytosolic free calcium concentration ($[Ca_i^{2+}]$), and transmembrane potential (MP).

Chemotaxis and migration are the initial responses of phagocytes to activation. They are characterized by initial adhesion processes, reorganization of the cytoskeleton and involvement of chemokine receptors. The binding of uncoated or opsonized particles to specific surface receptors initiates phagocytosis. Equipped with pattern recognition receptors, these cells can bind opsonized cells as well as specific components of the cell membrane. The receptors characterized best are opsonin-dependent receptors such as immunoglobulin receptors FcγRI, FcγRII, FcγRIII, complement receptor-3 and integrin receptors $\alpha_5\beta_1$ (VLA-5) and $\alpha_v\beta_3$ and opsonin-independent

receptors such as the N-formyl-Met-Leu-Phe (fMLP) receptor, mannose receptor, β-glucane receptor and CD14 (lipopolysaccharide receptor) [2].

The initial event induced by the binding of ligands is the accumulation and association of receptors on the cell surface. This enhanced presentation of receptors is a major property of phagocyte activation and includes signal transduction with, e.g., tyrosin kinases, GTPases and GTPkinases. Phosphatidylinositol 3-kinase is particularly important for the response of phagocytes to chemical signals. These stimulus-dependent activation steps can be significantly enhanced by priming (prestimulation). Priming is induced by treatment with low concentrations of agonists such as interleukin-8 (IL-8), lipopolysaccharide, leukotriene B4 and platelet-activating factor. The priming of neutrophils accelerates responses such as the production of reactive oxygen species. The last step of the activation cascade of phagocytes is the intracellular destruction of microorganisms and ingested foreign materials.

Dysfunction of phagocytes and the innate defense system can be congenital or acquired. Congenital defects can affect phagocyte functions such as chemotaxis, motility or adhesion. Examples are Chediak-Higashi syndrome, Schwachman's syndrome and chronic granulomatous disease. Cellular defects include gp110 defect, deficient endocytosis and cytotoxicity, and defects in myeloperoxidase, gp150 or G6-PD. The more frequent acquired immune defects include chronic diseases such as diabetes, autoimmune diseases, kidney or liver dysfunctions, alcoholism, immunosuppression during surgery, burns and viral infection. In these syndromes a reduced immune response is associated with increased susceptibility to infections and recurrent infections. By contrast, exaggerated responses of neutrophils, e.g. during systemic inflammatory response syndrome, may also lead to tissue destruction even in the absence of infection.

Abnormalities of the ligand-induced oxidative burst or phagocytosis reaction (see 'Oxidative Burst, Phagocytosis and Expression of Adhesion Molecules', pp. 343) as well as of changes in pH_i, $[Ca_i^{2+}]$ or MP can be indicators of decreased or abnormally increased responses of phagocytes [3–5].

Basic Principles of the Cytometric Analysis of Cell Physiological Responses

Cytometric methods (flow- or slide-based cytometry) are standard assays for the analysis of $[Ca^{2+}]_i$, pH and MP of cells. Other assays enable the estimation of additional intracellular ions such as K^+, Na^+, Cl^-. By cytometry, many individual cells can be measured with high throughput. The results are obtained in a standardized manner by establishing a calibration curve and analyzing the responses to defined stimuli. Fluorescent dyes that are sensitive to changes in cellular ion concentration, pH or MP enable cost effective measurements. All reagents mentioned hereafter are available from Invitrogen Inc./Molecular Probes (Eugene, OR, USA) and partially from other providers.

In general, cell physiological analyses are performed on living cells as measurements of time-dependent responses. The analyzed responses such as stimulation-induced changes in [Ca_i^{2+}] or MP are often rapid, and time may be recorded as an additional parameter during flow-cytometric data acquisition. Following a prerun with unstimulated cells, the sample is taken from the cytometer, the stimulus is added, mixed, and measurements are continued. Alternatively, the stimulus can be directly injected during the measurement [3, 4, 6] or can be placed in a drop of liquid above the cell suspension and mixed with the cells after the prerun by vortexing, a procedure which requires some dexterity. In case of sustained responses, the analysis may also be performed at a defined time after stimulation or as an endpoint determination. Negative controls using the solvent in the absence of a stimulus should always be included. Such negative controls are necessary because mechanical stimulation by addition of a stimulus and mixing may by themselves lead to cell activation in some cell types [6]. For data analysis, the time course of the cell physiological changes is determined and the data are calibrated using a calibration curve. Calibration, e.g. of fluorescence ratios, can be performed directly using specific software packages available as commercial products or for free.

Cell physiological analysis requires gentle standardized cell treatment. Staining, storage, and measurement should always be done under identical conditions. Changes in temperature should be avoided as these can stimulate cells [7]. Cells should not be stored on ice. Ideally, they should be measured at 37 °C if enabled by the instrument. Damaged and 'dead' cells can impair the measurements as such cells may suggest unresponsive cell populations. Dead cells can be discriminated from live cells using propidium iodide (PI) or 7-amino-actinomycin D (7AAD) (both at 5 μg/ml final concentration). Using a blue light source for excitation, these dyes enable clear distinction of dead cells as extremely bright cells with orange or red emission. Other dead-cell dyes are available if dead cells need to be discriminated using a different excitation source. The use of calcium chelators such as EDTA or citrate should be avoided. Keeping cells in calcium-free medium is not physiological. Intracellular calcium pools are depleted, and cells may respond less well to the same stimulus. In general, cells can be simultaneously stained for a cell physiological parameter and for cell surface antigens using antibodies. This multiparametric staining can yield useful additional information on the response of cell subsets. However, it should be remembered that the binding of antibodies to surface antigens may by itself lead to cell activation or modify cellular responses. For example, anti-CD3 will stimulate a calcium flux in T cells. If this type of analysis has to be performed, appropriate control experiments need to be done.

There are two principles for dyes to indicate changes in cell physiology. Indicator dyes can either change their fluorescence intensity (example: following an increase in intracellular free calcium, the dye Fluo3 increases in fluorescence whereas Fura Red fluorescence decreases) or their spectrum of emission (example: Indo-1). In general, ratiometric dyes that change their spectrum of emission (i.e. color) are best

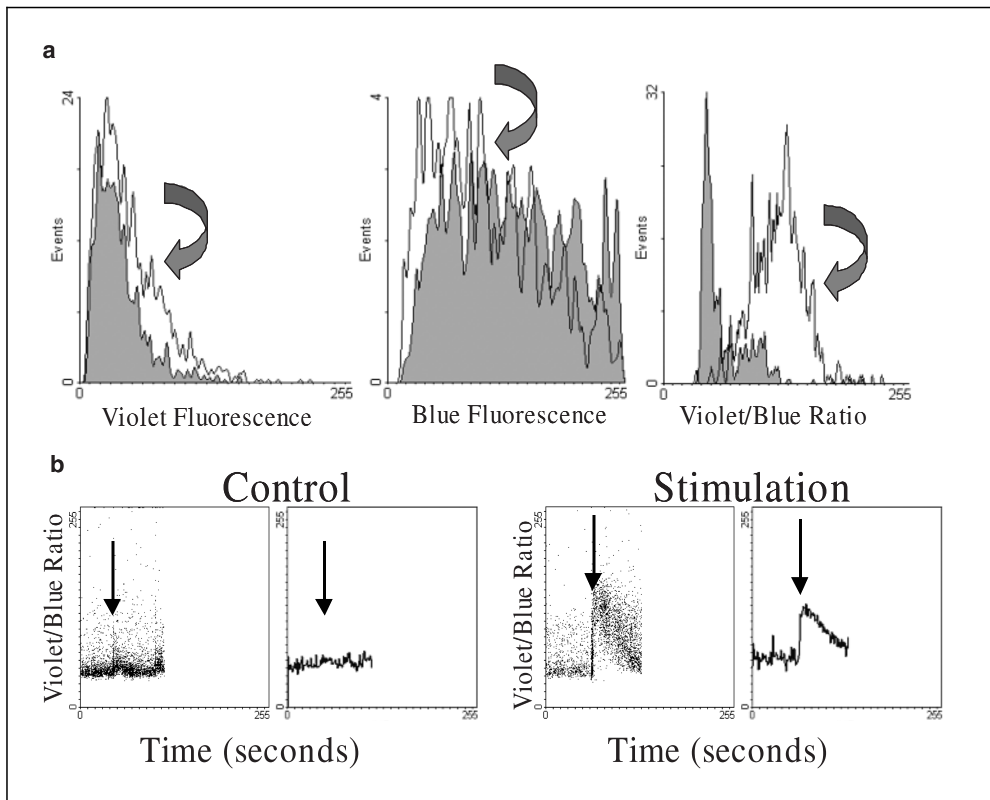

Fig. 1. Measurement of $[Ca^{2+}]_i$ with Indo-1.
a Unstimulated cells (gray) and stimulated cells (white) stained with Indo-1 are shown in their respective violet and blue fluorescence. Upon calcium release (straight lines) stimulated cells (gray shaded) show a slight shift towards violet and a reduction of their blue fluorescence. An unequivocal discrimination between responding and nonresponding cells is only possible using the violet to blue ratio (top right).
b Time course of a measurement following addition of a buffer solution as a control (left) or a stimulus (right). Arrows indicate the time point of the injection of the stimulus into the cell suspension during the measurement [22]. Following stimulation, the violet to blue ratio is dramatically increased. This leads to the clear discrimination of responding and nonresponding cells. Data are shown pairwise as dot plots or as mean calcium concentrations (lines).

suited for the measurement of cell physiological parameters. By the simultaneous measurement of two emission wavelengths (colors) the intensity of both colors is determined ratiometrically. This approach enables a calibrated analysis of different cells also in case of differences in dye loading (fig. 1). To this end, the cytometer should be able to record on line or analyze fluorescence ratios off line as additional parameters.

For the measurement, cells have to be loaded with the appropriate indicator. Labeling for MP is passive, i.e. after addition, the dye associates to cells shortly before the measurement. In contrast, staining for other dyes depends on active intracellular

enrichment through metabolic activity. These dyes include, among others, indicators for Ca^{2+} and pH. As they are mostly hydrophilic in their active state, i.e. when they can bind ions, they cannot penetrate the hydrophobic (lipophilic) cell membrane and accumulate in the cell. In order to label cells, these dyes are usually added as acetoxymethyl ester (AM). Due to ester groups, the dyes are lipophilic and can pass the cell membrane. Inside the cells these ester groups are removed hydrolytically by intracellular esterases. Thereby, the dyes are converted into their active forms and become hydrophilic. As hydrophilic dyes cannot escape from the cells, they accumulate there. Dye accumulation can be increased by applying a nonionic detergent, such as Pluronic F127. If the cells lack intracellular esterases, the dye does not accumulate due to the absence of hydrolysis. In this case, low cellular fluorescence does not change following stimulation. If this is the case, the active dye (without AM) can be used. Pluronic F127 or hypotonic shock may enable loading of the cells with the dye. Effective dye loading should be verified using a potent or maximal stimulus.

Principles of Measurement

Intracellular Free Calcium Concentration

Ca^{2+} is a universal second messenger. It appears in the cell at increasing concentrations only fractions of seconds to seconds after cell activation. In quiescent cells, calcium concentration is actively maintained at a level of 100–150 nmol/l by intracellular Ca^{2+}-dependent ATPase. By contrast, the concentration of Ca^{2+} in the physiologic extracellular space is as high as 1.3 mmol/l. Stimulation of surface receptors typically leads to a three-step increase in the calcium concentration of nonmuscular cells. In the first step, the sources of calcium release are calciosomes (intracellular calcium pools). In the second phase, the calcium increase depends on an influx from the extracellular space. This second phase is followed by an increase in the calcium level in the calciosomes and the export of membrane bound Ca^{2+} through the cell membrane with membrane-bound ATPase. This export leads to a reduction in $[Ca_i^{2+}]$.

Ca^{2+}-mobilized signals are regulated by the following mechanisms:

i) the intracellular second messengers inositol-1,4,5-triphosphate, cyclic ADP-ribose and nicotinic acid dinucleotide phosphate;
ii) Ca^{2+} channels in the membrane of the endoplasmatic reticulum or in the plasma membrane, and
iii) calcium sensors, including the ubiquitous calcium-binding protein calmodulin.

$[Ca_i^{2+}]$ can be intracellularly determined by a shift of Indo-1 fluorescence from blue (absence of calcium) to violet (presence of calcium). This method enables precise quantification independent of cell size and total fluorescence intensity as

Table 1. Calcium indicators in cytometry

Indicator	Emission response upon Ca^{2+} increase	Excitation wavelength nm	Emission wavelength nm	Calcium affinity K_d, nmol/l	
				22 °C	37 °C
Indo-1	ratio increase	325–360	390/520	~230	~250
Fluo-3	iIncrease	488	526	~330	~860
Fluo-4	increase	488	516	~350	n.i.
Calcium Green-1[a]	ncrease	488	530	~250	n.i.
Calcium Green-2	n.i.	488	536	~550	n.i.
Calcium Green-5N	n.i.	488	532	~14,000	n.i.
Calcium Orange	increase	550	575	~330	n.i.
Calcium Crimson	increase	550	610	~200	n.i.
Oregon Green 488 BAPTA-1[b]	n.i.	488	523	~170	n.i.
Oregon Green 488 BAPTA-2 [c]	n.i.	488	523	~580	n.i.
Oregon Green 488 BAPTA-5N[b]	n.i.	488	521	~20,000	n.i.
Fura Red[c]	ratio increase	405/488	597	~400	n.i.
Fluo-3/Fura Red[d]	ratio increase	488	530/660[e]	~400	n.i.

n.i. = No information.

[a] Calcium Green-1 fluoresces stronger than Fluo-3; both contain Ca^{2+} in a bound and unbound form. The effect of unbound Ca^{2+} for the increase in fluorescence is greater for Fluo-3

[b] Molar absorption of Oregon Green BAPTA-indicators at 488 nm is approximately twice as high as for the respective Calcium Green-indicator.

[c] Fura Red can be used ratiometrically regarding excitation, showing an increase in emission at 597 nm with calcium at 405 nm excitation and a decrease at 488 nm excitation.

dSimultaneous staining with Fluo-3 and FuraRed can be used for ratiometric measurement with blue excitation.

[e] Emission maximum of Fura Red is at 597 nm. In order to minimize spectral overlap between Fluo-3 and Fura Red, it is recommended to detect Fura Red fluorescence in the deep red channel.

the ratio of violet to blue fluorescence in the cells represents the calcium concentration. A calibration curve can be constructed using cells in a defined extracellular calcium concentration and the presence of calcium ionophores such as ionomycin or the brom/calcium ionophore A23187. Intracellular buffering of calcium can impair this calibration.

The kinetics of the $[Ca_i^{2+}]$ response to a stimulus is determined in three steps:

i) preparation of cells with Indo-1 and measurement at 37 °C without stimulation;
ii) addition of a stimulus and measurement of the changes in the calcium concentration until an equilibrium is reached, and

Table 2. Indicators for ratiometric measurement of the pH in cytomery

Dye	pK_a	Fluorescence, nm	
		excitation	emission
Fluorescein diacetate (FDA)	6.3	ratio 436/495	525
Carboxyfluorescein diacetate (CFDA)	6.4	ratio 441/488	535
Bis-carboxyethylcarboxyfluorescein-acetox methyl ester (BCECF-AM)	7.0	ratio 439/490	535
		488	ratio 520/620
Hydroxycoumarin (4-methylumbelliferone; 4-MU)	7.8	~350	ratio 430/470
		~350	ratio 450/560
Diacetoxydicyanobenzene (ADB) (yields dicyanohydrochinone (DCH) after hydrolysis)	8.0	~350	ratio 425/540
Carboxy SNARF-1 acetoxymethyl ester	7.5	488, 514 or 530	ratio 575/670

iii) addition of ionomycin or brom/calcium ionophore A23187 in order to determine the maximal Indo-1 ratio (positive control).

If ultraviolet excitation (365 nm) is not available, the indicators Fluo-3 or Fluo-4 can be used with blue excitation. This method also enables the sensitive detection of a calcium increase, and can be used in a ratiometric manner when combined with Fura Red. Additional fluorescent dyes to determine the calcium concentration are shown in table 1.

Alkalization of the Cytosol

The pH of eukaryotic cells varies in the range of 7.0–7.4. Regulation of the pH strongly correlates with leukocyte function. In phagocytes, intracellular pH also differs in various cell organelles. Furthermore, pH regulation is strongly linked to cell viability and apoptosis. The best-known mechanism of pH regulation is via amiloride-sensitive Na^+/H^+ transport. Alkalization of the intracellular pH is obtained by stimulating this antiport, e.g. with cytokines. Regulation of the intracellular pH is of particular importance for the activation of cells, as an increase in the intracellular pH indicates elevated metabolic activity.

The ratiometric indicator SNARF-1 is the dye of choice to measure cytoplasmic pH. The dye is excited by a blue laser and shifts in emission from green to red with increasing pH. Therefore, fluorescence is simultaneously measured in the green and the red fluorescence channels. pH changes are determined based on the red/green fluorescence intensity ratio. More pH indicators are shown in table 2. Ratiometric measurements with other indicators, e.g. FDA, CFDA or BCECF, are possible by determining the green fluorescence intensity following excitation at two different

wavelengths. These are frequently used in microscopy but are difficult to use in flow or slide-based cytometry.

Lysosomal Proteinases

Cellular endopeptidases are classified into four groups based on their inhibitors [8]:
i) serine proteinases, inhibited by diisopropyl fluorophosphate (DFP);
ii) cysteine proteinases, inhibited by E-64;
iii) asparagine proteinases, inhibited by pepstatin;
iv) metalloproteinases, inhibited by phenanthroline.

Due to their broad substrate specificity, these enzymes are involved in intracellular protein turn-over as well as extracellular tissue destruction during inflammation. Lysosomal proteases are differentially expressed in various cell lines and cell types. Therefore, they are relevant for cell typing. One specific function of phagocytes is the activity of elastase linked to the lysosomal destruction of bacteria. Extracellular release of elastase by phagocytes is associated with tissue destruction. Cysteine proteases expressed by monocytes and macrophages play an important role in antigen presentation.

The activity of lysosomal proteases can be detected intracellularly using R110 peptide derivatives. To this end, cells are incubated with the appropriate substrates, e.g. $(Z\text{-Arg-Arg})_2$-R110. The specificity of these methods is verified by preincubation with specific inhibitors, e.g. Z-Phe-Ala-CHN_2 for cathepsin B and L [9, 10].

Membrane Potential

Quiescent cells maintain large ion gradients between the intra- and extracellular space. As an example, potassium ions are accumulated in the cell via the activity of Na^+K^+ATPase. The efflux of K^+ ions establishes an electron counter-gradient and the cytoplasm becomes electron negative as compared to the extracellular medium. This electrochemical K^+ gradient is the major contributor to the MP of mammalian cells. The maintenance of a large negative MP was found to be a control mechanism that keeps cells in a resting state. In addition, metabolically active cells maintain a strong MP across the mitochondrial membrane system. The binding of ligands to transmembrane receptors in various cell types leads to rapid changes in the MP and subsequently to a physiological cell response.

The detailed investigation of MP in small cells became possible after the development of MP-sensitive indicator dyes (table 3). These probes are charged lipophilic molecules. They migrate between cytosol, cell membrane, and the extracellular medium according to the Nernst equation. For cationic (positively loaded)

Table 3. Indicator dyes for MP

Indicator	Excitation wavelength[a], nm	Emission wavelength[a], nm
Carbocyanines (mitochondrial membrane selective)		
$DiOC_2(3)$	488	500–510
$DiOC_5(3)$	488	520–530
$DiOC_6(3)$	488	520–530
$DiIC_1(3)$	488	575–585
$DiIC_1(5)$	633–647[b]	660–680
$DiIC_5(3)$	488, 514	540–580
$DiSC_3(5)$	568, 633	$>590 > 680$
JC-1[c]	488	527/590
Oxonols (plasma membrane selective)		
$DiBAC_4(3)$	488	520–530
$DiBAC_4(5)$	568–595	610–640
$DiSBAC_2(3)$	568	590–630
$DiTBAC_4(3)$	488	575–585
$DiTBAC_4(5)$	633–647	670–680
Rhodamines (mitochondrial membrane selective)		
Rhodamine 123[c]	488	530
CMTMRos[c, d]	488	600
CMXRos[c, d]	488	600

[a] Common laser and emission regions.
[b] Depending on laser.
[c] Mitochondrial MP.
[d] Potential measurement also possible after fixation of cells.

indicators such as cyanine dyes, cellular concentration decreases when the cell depolarizes (i.e. MP drops to zero) and increases when the cell hyperpolarizes (becomes negative). Using the negatively charged oxonol dye, the response is in the opposite direction.

MP indicators for flow-cytometric analysis can be divided into three major types: carbocyanine, oxonol and rhodamine/rosamine dyes (table 3). All of them respond to MP changes by alterations in their transmembrane distribution. They can be used to measure changes in the MP of nonexcitatory and stimulated cells induced, e.g. by respiratory activity, ion channel permeability, receptor activation, or binding of substances with pharmacological activity. With some limitations, within cells oxonols generally respond to the plasma MP while carbocyanine and rhodamine dyes respond to mitochondrial MP differences in a concentration-dependent manner. [11].

Carbocyanines

Membrane hyperpolarization leads to increased intracellular uptake of moderately lipophilic cationic carbocyanine dyes such as $DiOC_6(3)$ and $DiOC_5(3)$. Protocols for flow cytometry apply low extracellular dye concentrations (<0.1 μmol/l) in order to minimize the toxic effect of carbocyanine dyes [11, 12]. Staining at low extracellular dye concentrations mainly reflects the mitochondrial MP, and to a lesser extent the plasma MP [12, 13]. Potential-dependent enrichment of the carbocyanine dye JC-1 in mitochondria leads to a pronounced shift in its emission wavelength (from ~527 to 590 nm) due to formation of J-aggregates [14]. The ratio of green to red JC-1 fluorescence at 488 nm excitation can be used to determine the mitochondrial MP [15]. Alternative dyes include $DiOC_2(3)$ (blue excitation) and $DiIC_1(5)$ (red excitation). Loss of mitochondrial MP gradients is a very early step in the cascade of programmed cell death (apoptosis). Thus, JC-1 is a good indicator of apoptosis.

Oxonols

Oxonols like $DiBAC_4(3)$, the most popular of these dyes in flow cytometry, are lipophilic anions. Hyperpolarization of the membrane leads to reduced cell staining by oxonols. This is in contrast to the increase observed using cationic carbocyanines. Oxonols are more specific indicators of the plasma MP than carbocyanines. Because of their anionic character, no labeling of mitochondria occurs. For the same reason oxonols are substantially less toxic than carbocyanines. In addition, oxonols are better suited to detecting the viability of bacteria than carbocyanines and rhodamines [16].

Rhodamine and Rosamine

Rhodamine 123 is a lipophilic cation and is particularly well suited for the labeling of mitochondria in living cells [17]. Its spectral properties resemble those of fluorescein. As labeling is dependent on mitochondrial function, staining intensity directly reflects cell viability [18]. Furthermore, it enables discrimination of cell populations based on their level of mitochondrial respiratory activity [19, 20]. The rosamine derivatives CMTMRos and CMXRos are also suitable for measuring mitochondrial MP. Both dyes bind to intracellular proteins. Fixation after stimulation therefore enables one to retain the staining of cells.

Protocols

Isolation of Leukocytes

For the determination of cell physiological parameters, gentle primary isolation of leukocytes from erythrocytes is preferred over secondary erythrocyte lysis.

Work Steps

- Pour whole blood into heparin tubes (10 U/ml heparin; do not use EDTA or citrate tubes as cells may not respond physiologically following calcium depletion).
- Overlay 5 ml Ficoll (density: 1.077 g/ml) carefully with 3 ml blood. Avoid mixing of blood with Ficoll (Ficoll activates leukocytes).
- Keep samples for approximately 40 min at room temperature; erythrocytes aggregate and sediment into Ficoll; leukocytes remain above the layer and can be seen as an opaque zone.
- Carefully withdraw approximately 800 μl of the supernatant with leukocytes. Avoid contact with Ficoll.

The cell suspension thus obtained will contain $\sim 2 \times 10^7$ unseparated leukocytes/ml in autologous plasma.

If necessary, Ficoll separation can be avoided. In this case, leukocytes are specifically labeled and thus discriminated from abundant erythrocytes during analysis. Antibodies (CD45, pan-leukocyte antigen), a viable dye for DNA (Hoechst 33258 (excitation: UV, emission: 490 nm); SYTO 16 dye (excitation: 488 nm, emission: 518 nm) or DRAQ5 (excitation: 488 nm or 633 nm, emission: 670 nm) can be used for this purpose. One has to make sure that these additional dyes do not lead to artifactual stimulation or modify the response to a stimulus. Thus DRAQ5 is at present the only cell permeant DNA dye that is excitable by a red light source. Unfortunately, it has some toxicity and may thus alter physiological responses.

Measurement of Intracellular Free Calcium Concentration

Staining

Indo-1

- 5×10^6 cells/ml are incubated in HEPES-buffered medium (containing a physiological concentration of Ca^{2+}) with 0.5–5 μmol/l Indo-1/AM at 37 °C (water bath). Staining is completed after 20–30 min. Depending on cell type, the lowest possible concentration that results in a homogeneous fluorescence ratio should be selected.
- Add PI at a final concentration of 5 μg/ml. Assess the fluorescence of Indo-1/calcium complexes by using band-pass filters in the 390- and 440-nm range. For the PI fluorescence of dead cells, a 620-nm band-pass filter is required. Both dyes are excited by a UV light source (~360 nm).

Fluo-3/AM

- 5×10^6 cells/ml are incubated in HEPES-buffered medium (containing a physiological concentration of Ca^{2+}) with 0.5–2 μmol/l Fluo-3/AM at 37 °C (water bath). Staining is completed after 20–30 min. Depending on cell type, the low-

est possible concentration that yields homogeneous fluorescence should be selected.
– Add PI (final concentration 5 μg/ml). Assess Fluo-3 fluorescence using a 535-nm band-pass filter (excitation: 488 nm); PI fluorescence: 620-nm band-pass filter.

Measurement
– Perform an initial measurement of the unstimulated cells for 30 s in order to determine the basal value.
– Add a stimulus, e.g. fMLP (10^{-8} mol/l).
– Negative control: addition of the solvent of your stimulus.
– Positive control: addition of ionomycin or Br/Ca ionophore A23187 (2 μmol/l) for a maximal calcium response.
– Continue the measurement for a few minutes after stimulation depending on the kinetics of the response; acquire time as an additional parameter.

The absolute [Ca_i^{2+}]can be determined using a calibration curve generated with known extracellular calcium concentrations [21, 22]. This is substantial extra work, however.

Data Analysis
– Data (ratio for Indo-1 or intensity for Fluo-3) are plotted as dot plots versus side scatter. The leukocyte subsets of neutrophils, monocytes and lymphocytes can be discriminated based on scatter.
– Gate on the cells of interest (e.g. neutrophils) and show their PI fluorescence as a histogram. Gate on the PI-negative (viable) cells.
– Show the Indo-1 ratio (violet/blue) or the Fluo-3 fluorescence intensity vs. time. Set a cut-off for a positive reaction based on the negative control. Cells with values above this limit are regarded as responding.
– Determine the percentage of responding cells after stimulation. Positive control should be 100%.
– The mean calcium concentration (absolute values if a calibration curve has been established, otherwise relative values) can now be reported as a function of time. This can be performed with commercial and shareware software.

Intracellular pH

Staining
SNARF-1
– 5×10^6 cells/ml are incubated in HEPES-buffered medium with 0.2–1 μmol/l SNARF-1/AM at 37 °C (water bath). Staining is completed after 20–30 min. Depending on cell type, the lowest possible concentration that yields a homogeneous fluorescence ratio should be selected.
– Add PI (final concentration 5 μg/ml).

Measurement

- For the orange SNARF-1 fluorescence select a band-pass filter of 575 nm; for the red SNARF-1 and PI fluorescence, a 620-nm long-pass filter. (Although both PI and SNARF-1 red are measured in the same fluorescence channel, PI fluorescence intensity is orders of magnitude higher and easily distinguishable. Dead cells will show low or no SNARF-1 orange fluorescence.)
- If feasible on your instrument, also directly measure the SNARF-1 red/orange ratio. Turn on time as a parameter.
- Initial baseline measurement for 30 s without stimulation.
- Add a stimulus, e.g. fMLP (10^{-8} mol/l), or TNF-α (1 ng/ml) for neutrophils.
- Negative control: addition of the solvent of the stimulus.

Calibration Curve

- After SNARF-1/AM incubation, split sample into six aliquots (see staining 1).
- Centrifuge ($60 \times g$, 4 °C, 5 min).
- Resuspend and incubate for 5 min in 140 mmol/l KCl buffer (10 mmol/l Mes, 10 mmol/l HEPES, pH 6.4; 6.8; 7.2; 7.6; 8.0; 8.4; each with 10 μmol/l nigericin).
- Measure.

Data Analysis

- See protocol for calcium.
- When a calibration curve is measured, the SNARF-1 ratio values can be transformed into absolute pH values.

Proteinases

Staining

- Incubate 5×10^6 cells/ml in HEPES-buffered medium with or without specific proteinase inhibitors (e.g. 100 μmol/l Z-Phe-Ala-CHN_2 or 1 mmol/l DFP) at 37 °C for 10 min.
- 20 min incubation with appropriate proteinase substrate, e.g. 4 μmol/l $(\text{Z-Arg-Arg})_2$-R110.
- Add PI (final concentration 5 μg/ml).
- Assess R110 fluorescence with a 515- to 535-nm band-pass filter; PI fluorescence, e.g. with a 620-nm long-pass filter (excitation: 488 nm).

Measurement and Analysis

The intracellular turnover is determined by the amount of fluorescence acquired during the incubation.

Membrane Potential

Staining
JC-1

- Transfer 5×10^6 cells/ml into cell culture medium with 10% serum and mix with JC-1 (final concentration: 20 μmol/l; note: titration is recommend as ratiometric response very much depends on JC-1 loading into cells.). Mix thoroughly during addition and the following 20 s.
- Wash twice with PBS; centrifuge for 5 min at $200 \times g$.
- Incubate cells for 15 min at room temperature.
- Add PI (final concentration 5 μg/ml).

$DiBAC_4(3)$

- Transfer 5×10^6 cells/ml into HBSS. Incubate with $DiBAC_4(3)$ for 10–20 min at 37 °C (final concentration: 100 nmol/l). Staining is completed after 10 min. Depending on cell type, select the lowest possible concentration yielding homogeneous fluorescence.
- Add PI (final concentration 5 μg/ml).

Measurement

- For JC-1, use 530-nm (green) and 570-nm (orange) band-pass filters; for $DiBAC_4(3)$, a 525-nm band-pass filter, for PI fluorescence a 620-nm long-pass filter (excitation: 488 nm).
- Initial baseline measurement for 30 s without stimulation.
- Add a stimulus, e.g. fMLP (10^{-8} mol/l), or TNF-α (1 ng/ml) for neutrophils.
- Negative control: addition of the solvent of your stimulus.
- Positive control: addition of valinomycin (10 μmol/l in ethanol); valinomycin is an ionophore and leads to cell depolarization.

Calibration Curve

- Stain 250 μl cell suspension for 10 min at 37 °C in HBSS with 250 μl $DiBAC_4(3)$ solution (400 nmol/l) and 500 μl HBSS or K-HBSS (K^+ concentration in 5 mmol/l steps from 5 to 37.5 mmol/l).
- Measure fluorescence.

Data Analysis

- See protocol for calcium.
- By means of a calibration curve, the measured $DiBAC_4(3)$ values can be converted to absolute MP values according to a modified Nernst equation:

$$E_K + (\text{mV}) = -59 \log [K^+]_i/[K^+]_o \,. \tag{1}$$

$E_K + (\text{mV})$ is the K^+ MP, the intracellular K^+ concentration $[K^+]_i$ is estimated at 100 mmol/l [3], $[K^+]_o$ is the concentration of the extracellular potassium of the cali-

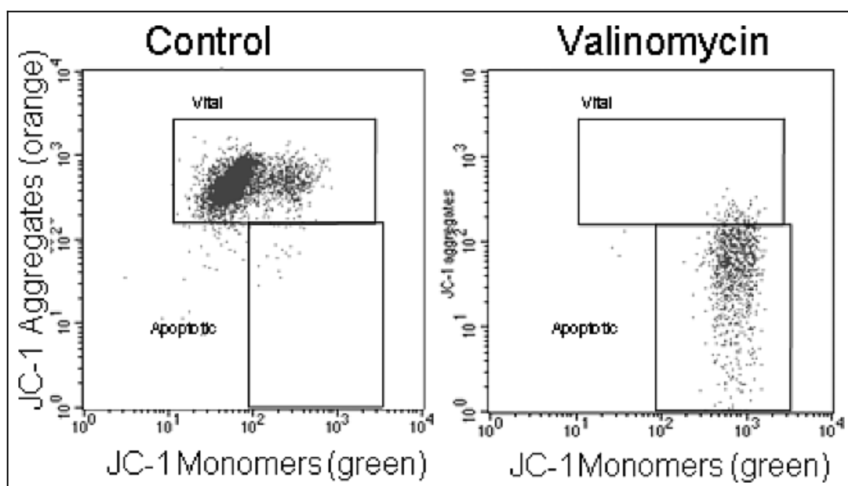

Fig. 2. Measurement of the MP with JC-1. Left figure shows a control measurement without stimulation. Right figure depicts stimulation with valinomycin (2 μmol/l). Valinomycin leads to cell depolarization and apoptosis.

bration solution. In the range of 5–40 mmol/l extracellular K^+ the DiBAC4(3) fluorescence intensity and the E_K should have a linear relationship.

Expected Results

A transient increase in $[Ca_i^{2+}]$ is typically achieved with low concentrations of fMLP (see typical examples for maximum response in figures 1 and 2, and in Tárnok et al. [3, 5, 6]. However, low concentrations are insufficient to evoke an oxidative burst. These experiments are of some use for determining the reaction of blood cells to a specific ligand, but are not well suited for dose-response determinations.

Determination of intracellular elastase activity in neutrophils or of cathepsin B in monocytes yields information about altered cell maturation and differentiation cells. This type of information can be important for inflammatory processes or neoplastic diseases of the hematopoietic system. Increased cathepsin B activity correlates with activation of monocytes in vivo. Degranulation can be observed in vivo during inflammatory processes, but is hardly detectable in peripheral blood cells. One can assume that this process is associated with cellular adhesion.

Troubleshooting

Artificial activation of cells, such as spontaneous calcium burst or nonspecific labeling, are crucial problems in the analysis of phagocytic cells. Both problems can be minimized if samples are quickly and carefully obtained and rapidly analyzed.

Transient Calcium

- Insufficient staining with Indo-1 or Fluo-3 can be improved by the addition of Pluronic F-127.
- Minor changes in the Indo-1 fluorescence ratio (even after stimulation with calcium ionophore) can be due to incomplete hydrolysis of Indo-1/AM. Complete hydrolysis can be obtained by extended incubation in a substrate-free environment. Inactivation of the calcium ionophore can be an additional reason. In solution, the ionophore is only stable for a limited period of time (even at −20 °C). It should be stored in lyophilized aliquots at −80 °C until use.
- The lack of a cellular response can also be due to the absence of extracellular calcium. Check that the cells are not kept in a medium containing a calcium chelator. The lowest possible concentration of fluorochrome that yields homogeneous signals should be applied in order to avoid intracellular buffering of calcium by the probe.

Alkalinization of the Cytosol

The absence of a cellular response in the presence of pH-sensitive cellular fluorescence may indicate the simultaneous activation of hydrogen-exporting antiport and the metabolic generation of H^+ in the cells. Both processes can be separately addressed by the addition of the antiport inhibitor amiloride, which enables analysis of the metabolic generation of H^+ without the compensatory effect of the antiport.

Lysosomal Proteinases

Selective cell death may occur in samples incubated with proteinase substrates. At the same time addition of an inhibitor preserves cellular viability. A reason for this phenomenon can be a high intracellular metabolic accumulation of R110. This effect can be avoided by reducing the incubation time or the substrate concentration.

The sensitivity of fluorescent peptide substrates to enzymes depends on factors such as pH, ion strength or oxidative potential. Therefore, it is advisable to test the specificity of such experiments under unknown intracellular conditions with specific inhibitors.

Membrane Potential

Failure of cells to respond to stimulation may be due to staining toxicity. This may happen especially when using cyanine dyes. One should avoid prolonged cell incubation and reduce the dye concentration in a titration experiment. Absence of mito-

chondrial staining as checked by microscopy confirms a correct concentration range. In general, oxonol dyes are preferable for the measurement of cellular MP.

Summary

The analysis of cell physiological parameters such as pH, Ca^{2+} or membrane potential enables sensitive detection of functional responses to stimulation. The functional characterization of phagocytic blood cells is an interesting application. Neutrophils and monocytes are involved, among others, in acute and chronic inflammatory processes. Dysfunctions of these cells can be accompanied by increased susceptibility to infections. The functional repertoire of phagocytes correlates with their state of differentiation. Ligands, e.g. bacterial products, cytokines, complement, can activate phagocytes and induce specific cellular reactions like chemotaxis. Phagocytosis comprises a complex cascade of stimulatory actions and functional responses. Cellular activation also involves processes on the cell surface such as expression of adhesion antigens which determine an interaction with other blood cells or the endothelium. Artifactual activation can already occur during the preparation of neutrophils, and this can be demonstrated using sensitive cytometric assays.

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