methods

Leukocyte lipid bodies: inflammation-related organelles are rapidly detected by wet scanning electron microscopy

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Abstract Leukocyte lipid bodies are dynamic, functionally active organelles with central roles in inflammation. Here, we report that leukocyte lipid bodies are facilely detected by a versatile, potent technique, termed wet scanning electron microscopy (SEM), which combines the rapid preparation of light microscopy with the resolution of SEM. Using as leukocyte models resting and agonist-stimulated human eosinophils, cells that generate prominent numbers of lipid bodies in inflammatory conditions, we demonstrated that lipid bodies can be rapidly imaged as bright, highly contrasted structures under wet SEM and scored by computerized image processing. Critical advantages of this approach are that it permits cell observation in a fully hydrated system and facilitates lipid preservation. These attributes are especially important because lipid bodies are degraded during routine dehydration processes. Moreover, this technology is advantageous over lipophilic fluorescent probes because it allows sustained detection of lipid bodies in contrast to short-lived fluorescent labeling of these organelles. The value of wet SEM in enabling rapid and large-scale lipid body imaging and scoring within leukocytes is particularly important because lipid bodies are organelles underlying the heightened functions of inflammatory cells. Wet SEM technology provides new approaches and opportunities for delineations of lipid bodies in inflammatory diseases, including allergic inflammation.—Melo, R. C. N., A. Sabban, and P. F. Weller. **Leukocyte lipid bodies: inflammation-related organelles are rapidly detected by wet scanning electron microscopy. *J. Lipid Res.* 2006.** 47: **2589–2594.**

Supplementary key words lipid droplets • lipid imaging • eosinophils • wet samples

The organization of lipids bearing esterified arachidonic acid into distinct intracellular sites has particularly important roles in cells of the immune system (1). In leukocytes and other cells of the inflammatory response, arachidonyl lipids are often discretely organized within cytoplasmic lipid bodies and serve as precursors of inflammatory eicosanoid mediators (2–5). After in vitro inflammatory stimuli or during in vivo inflammatory diseases, including infectious diseases, the numbers and sizes of lipid bodies increase within inflammatory cells (e.g., neutrophils, eosinophils, and macrophages), and these organelles become predominant sites for the synthesis of eicosanoid inflammatory mediators (3, 6–8). Lipid bodies within inflammatory cells contain all of the enzymes necessary for eicosanoid synthesis, including cyclooxygenases, 5-and 15-lipoxygenases, and leukotriene C_4-synthase (3, 4, 6, 9). Both lipid body formation and the compartmentalization of enzymes within lipid bodies are highly regulated cellular events involved in the heightened capacity of leukocytes to generate eicosanoids. Lipid bodies, therefore, are dynamic, functionally active organelles that both contribute to the genesis of inflammatory and immune responses and act as targets for the control of inflammatory diseases.

Although lipid bodies have central roles in inflammation and are considered structural markers of inflammatory cells in a range of diseases (6, 10, 11), their identification has methodological limitations because lipid bodies dissipate upon drying or dissolve upon fixation and staining with alcohol-based reagents (12–14). For example, May-Grunwald-Giemsa staining causes the dissolution of lipid bodies (15). Usually, these organelles are identified by light microscopy using lipophilic fluorescent probes (12, 13, 16) or staining with osmium (17). At the ultrastructural level, lipid bodies can be observed by transmission electron microscopy (TEM) without any additional labeling, by which they appear as distinct organelles lacking delimiting classical trilaminar membranes (2, 10, 18). However, TEM preparations are time-consuming and require costly processing, such as embedding and thin sectioning.

Manuscript received 24 July 2006 and in revised form 29 August 2006.

Published, JLR Papers in Press, August 29, 2006.
DOI 10.1194/jlr.D600028-JLR200

Abbreviations: BODIPY, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate, diammonium salt; BSE, backscattered electron; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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Here, we report that leukocyte lipid bodies can be facilely detected by a wet scanning electron microscopy (SEM) technique, named WETSEM™, which enables the imaging of hydrated samples and combines the rapidity of preparation of light microscopy with the resolution of electron microscopy (19). Moreover, we have compared lipid body imaging by wet SEM with different cell preparations and staining methods for light microscopy. Using resting and agonist-stimulated human eosinophils, cells that can contain prominent numbers of lipid bodies (3), as leukocyte models, we show that the wet SEM technology provides new approaches that facilitate the study of lipid bodies in inflammation.

MATERIALS AND METHODS

Chemicals

Paraformaldehyde (16%; electron microscopy grade), glutaraldehyde (25%; electron microscopy grade), uranyl acetate, and OsO_4 were purchased from Electron Microscopy Sciences (Fort Washington, PA). Other chemicals, unless indicated, were obtained from Sigma (St. Louis, MO).

Eosinophil isolation, stimulation, and viability

Granulocytes were isolated from the blood of healthy donors as described (20), and eosinophils were purified by negative selection using human eosinophil enrichment cocktail (StemSep™; StemCell Technologies, Vancouver, Canada) and the magnetic activated cell sorter (MACS) bead procedure (Miltenyi Biotec, Auburn, CA). Sample procurement and experiments were approved by the Committee on Clinical Investigation, and informed consent was obtained from all subjects. Eosinophil purity was >99%. For different approaches, eosinophils (10^6 cells/ml) were stimulated with recombinant human eotaxin (100 ng/ml; R&D Systems, Minneapolis, MN) in RPMI-1640 medium plus 0.1% ovalbumin or medium alone at 37°C for 1 h (21). Cell viability after stimulation was >95%, as determined by ethidium bromide incorporation. A nonphysiological challenge with the calcium ionophore A23187 (0.5 μM) was also used in some experiments, as described (22). Alternatively, unstimulated eosinophils were studied in whole samples of white blood cells isolated from human blood. Briefly, blood (3 ml) was mixed in a 6% dextran solution (1 ml) containing 3% glucose and 0.9% NaCl plus 60 μl of 0.5 M EDTA. After 15 min of sedimentation, the leukocyte-rich plasma layer was recovered, mixed with PBS, and centrifuged. White blood cells were resuspended in PBS and prepared for wet SEM.

Sample preparation for wet SEM

Suspensions containing nonstimulated and stimulated leukocytes were immediately placed into individual capsules (QuantomiX, Ltd.) (15 μl of cell suspension/capsule) containing a thin, electron-transparent partition membrane. Leukocytes were allowed to adhere to the membrane for 15 min at room temperature. Within the microscope, this membrane isolates the content of the capsules from the vacuum and allows both the penetration of electrons and the collection of backscattered electrons (BSEs) while withstanding a pressure difference of one atmosphere (19, 23, 24). After adherence, cells were fixed with a freshly prepared mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS for 30 min and washed in the same buffer twice. OsO_4 staining was performed by washing the fixed samples in water and subsequently incubating them for 15–30 min in 0.5% OsO_4 in distilled water. After washing the samples several times with water, the capsules were sealed.

Sample preparation, staining, and imaging by light microscopy

Lipid bodies from unstimulated and eotaxin-simulated human eosinophils were imaged by light microscopy with different techniques. Cell preparation was done in two ways: using a cytocentrifuge (17) or by spreading a mixture of eosinophils with melted agarose matrix (Pierce, Rockford, IL) onto a slide (25). OsO_4 staining and lipid body enumeration were performed exactly as described (17) in both cytospin and agarose preparations. Briefly, slide preparations, while still moist, were fixed with 2% paraformaldehyde in Ca^{2+}-Mg^{2+}-free HBSS ($HBSS^{-/-}$), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained in 1.5% OsO_4 (30 min), rinsed in 0.1 M cacodylate buffer, immersed in 1% thiocarbohydrazide (5 min), rinsed with 0.1 M cacodylate buffer, restained with 1.5% OsO_4 (3 min), and then dried and mounted. Lipid bodies were enumerated using a 100× objective lens in 50 consecutively scanned cells. In some experiments, cytospin preparations were counterstained with the Hema 3® kit (Fisher Scientific, Houston, TX) after OsO_4 staining.

For imaging of fluorescently labeled lipid bodies, eosinophils in agarose preparations were covered with a perfusion chamber (CoverWell™; Grace Bio-Labs, Bend, OR) and incubated with 1 μM fluorescent acyl-modified phosphatidic acid, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate, diammonium salt (BODIPY® FL C_5-HPA; Molecular Probes, Eugene, OR) for 1 h at 37°C before fixation or with Nile Red (1:10,000 from a stock solution of 1 mg/ml in acetone) for 5 min at room temperature after fixation. Fixation was done with 2% paraformaldehyde in $HBSS^{-/-}$ for 5 min. After staining/fixation, cells were washed twice in $HBSS^{-/-}$, dried, mounted with Aqua Poly/Mount (Polysciences, Warrington, PA), and viewed on a Provis AX-70 Olympus microscope. Alternatively, $HBSS^{-/-}$-wet slides were analyzed after coverslipping. Analyses were performed with bright-field (osmium staining), phase-contrast, or fluorescence microscopy at 1,000×.

SEM imaging

Sealed capsules were placed on the specimen stage of the scanning electron microscope to be viewed in the wet state. After applying the vacuum, imaging was performed according to the operating instructions of the microscope, with attention given to some features that may differ from standard SEM imaging modes. First, imaging was done using a BSE detector. Second, the electron beam energy should be at least 15 kV, and typically 30 kV. Imaging is performed at higher current (spot size) and slower scan rate to compensate for the relatively low contrast. Because the capsule is taller than a standard specimen stub, the specimen stage must be adjusted to ensure an optimal working distance. The specific settings may vary between microscopes and between different specimens. The images presented in this work were obtained on a Leo 1450VP (Zeiss) or on a FEI XL-30 (Eindhoven, The Netherlands) scanning electron microscope with a working distance of 9 or 8 mm, respectively, beam energy of 15–30 kV, beam current of 200–800 pA, and scan rates of 1.3–120 ms/line at 484 lines/frame. Acquired images were evaluated for lipid body enumeration using Image Pro-Plus® software

(Media Cybernetics, Inc., Silver Spring, MD). Data were compared by Student's *t*-test ($P < 0.05$).

RESULTS

Lipid body visualization within human blood eosinophils

To identify lipid bodies as well as their distribution within entire eosinophils, freshly isolated human eosinophils were stimulated with eotaxin or medium alone for 1 h and immediately prepared for imaging with the wet SEM technique or by light microscopy. Eotaxin was chosen because this agonist is a potent eosinophil activator (26). Eosinophils activated by this agonist or other physiologic stimuli in vitro, like those recruited during inflammatory processes in vivo, exhibit various morphologic changes associated with activation (27), including the induction of new lipid body formation (7).

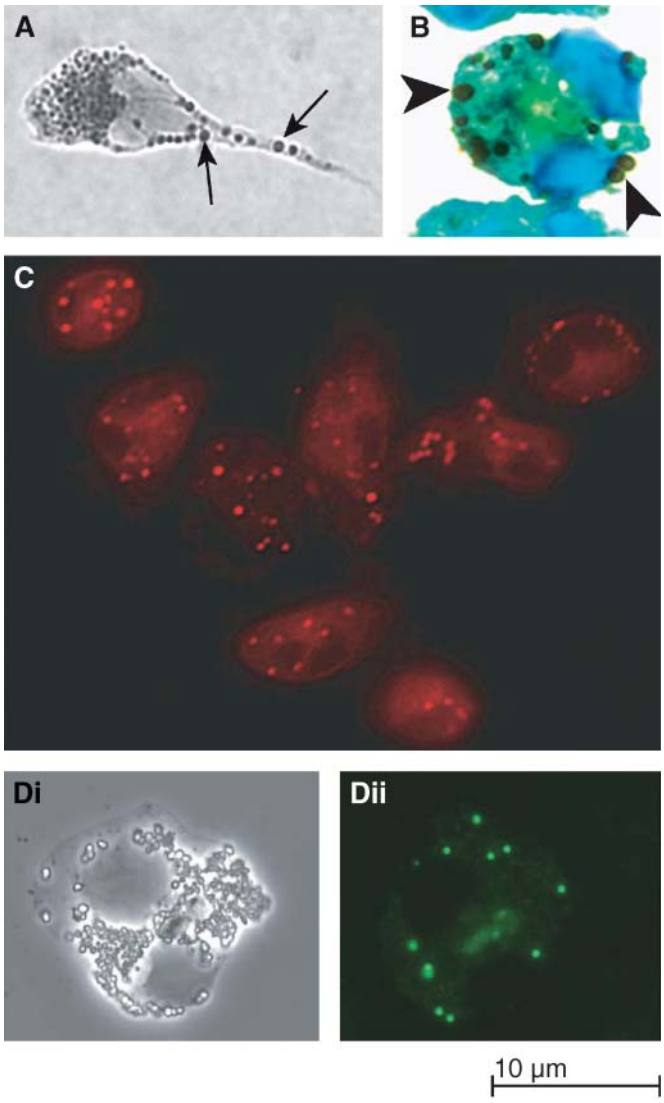

Fig. 1. Lipid bodies within human blood eosinophils imaged by light microscopy after staining with osmium (A, B), Nile Red (C), or 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate, diammonium salt (BODIPY) phosphatidic acid (D). Lipid bodies appear as round, dark (A, arrows; B, arrowheads) or fluorescent (C, D) organelles distributed throughout the cytoplasm. Di shows the identical field as Dii by phase-contrast microscopy. In B, cells were counterstained with Hema 3®. A and B show cytospin and agarose cell preparations, respectively. Eosinophils were obtained from the blood of normal human donors and stimulated with eotaxin (A, B), calcium ionophore (C), or medium alone (D) as described previously (22).

Light microscopy

By light microscopy, lipid bodies appeared as dark or fluorescent organelles in cell preparations stained with osmium (**Fig. 1A, B**) or fluorescent lipid probes (Fig. 1C, Dii), respectively. In general, cells kept in agarose showed better morphology compared with cells from cytospin preparations (compare Fig. 1A, B). Moreover, shape changes, a feature of activated human eosinophils (28),

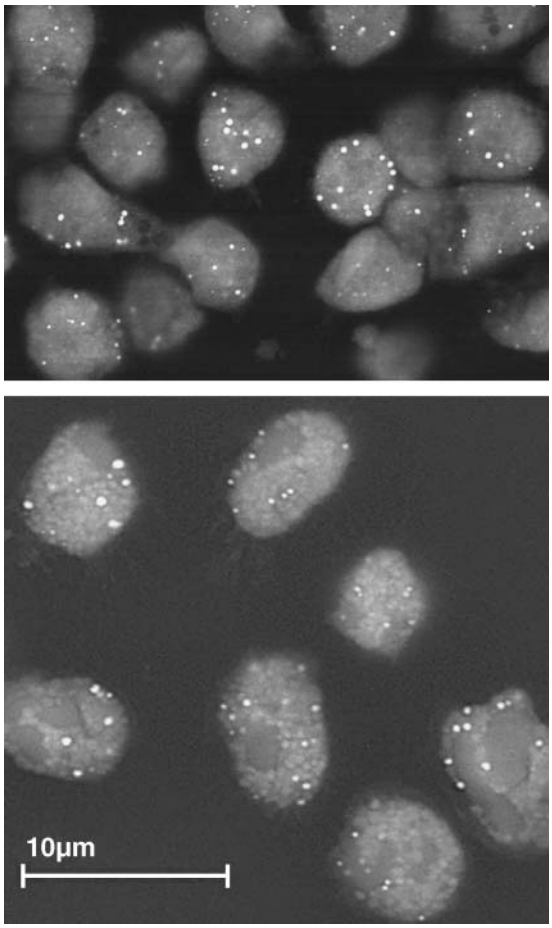

Fig. 2. Human blood eosinophils viewed with wet scanning electron microscopy (SEM). Lipid bodies appear as highly contrasted, round, and white electron-dense organelles. Eosinophils were obtained from the blood of normal human donors, isolated by negative selection, and stimulated with eotaxin (100 ng/ml for 1 h). Cells were stained with osmium followed by uranyl acetate, as described in Materials and Methods, for imaging by SEM under fully hydrated conditions.

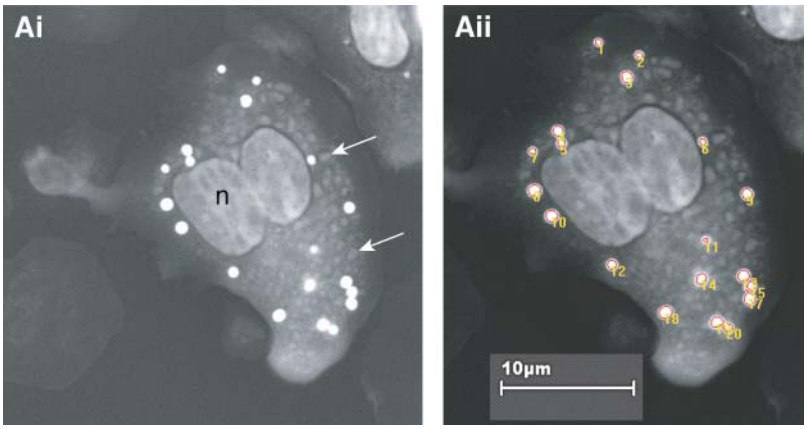

Fig. 3. Lipid body imaging and enumeration within a human blood eosinophil. Bright lipid bodies are observed in conjunction with numerous eosinophil-specific granules (arrows) within the cytoplasm. In Aii, lipid bodies were scored by computerized image processing. Eosinophils were visualized from a whole sample of white blood cells by wet SEM. Enumeration was performed using Image Pro-Plus® software. n, nucleus.

could be easily observed when the cells were embedded in an agarose matrix (Fig. 1A). Although lipid bodies were revealed by osmium (Fig. 1A, B, arrows and arrowheads), this staining induced a brownish background in the eosinophil cytoplasm, where most specific granules were concentrated (Fig. 1A). In eosinophils prepared with Nile Red (Fig. 1C) or BODIPY (Fig. 1D), lipid bodies were clearly observed as red or green spots, respectively, but the fluorescence was not stable enough to enable an accurate quantification of these organelles. Moreover, adequate detection of lipid bodies with both fluorescent stains was achieved only in wet slides. Samples dried at the end of the staining procedure did not show a good preservation of lipid bodies. Staining artifacts were also observed. These have arisen from cellular autofluorescence exhibited by damaged or dying cells (~5% of the cells), as observed previously in other approaches using fluorescent products (25). Quantitative analyses of lipid bodies stained with osmium showed an increased number of these organelles in eotaxin-stimulated cells [3.7 ± 1.5 lipid

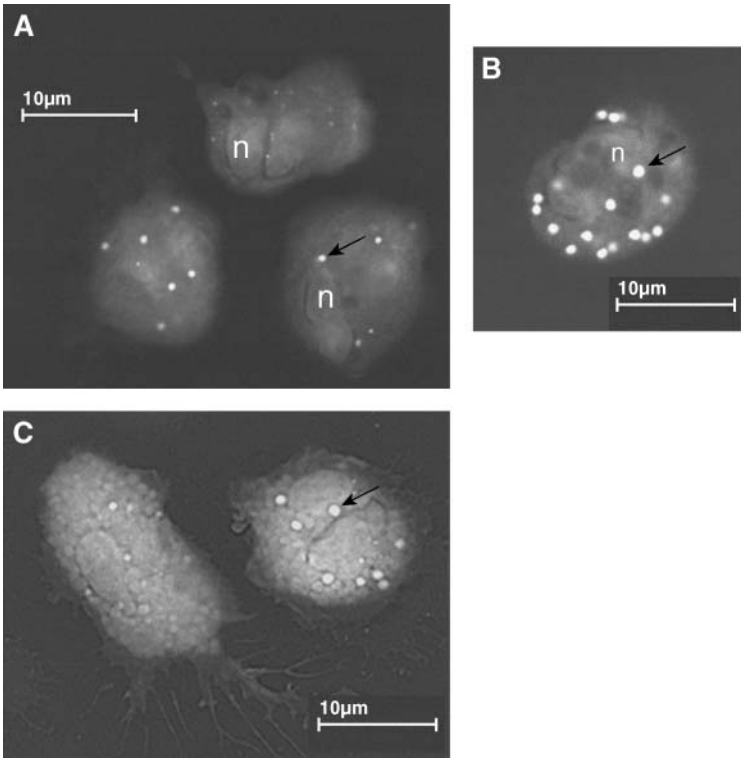

Fig. 4. Lipid body imaging within unstimulated (A) and eotaxin-stimulated (B, C) human eosinophils observed by wet SEM. Both the number and size of lipid bodies were apparently higher in stimulated than in unstimulated cells. Note the presence of lipid bodies in contact with the periphery of the nucleus (arrows). In C, a polarized eosinophil with pronounced cell extensions is shown. Hydrated eosinophils were prepared as described for Fig. 2 and imaged on a scanning electron microscope. n, nucleus.

bodies/cell in unstimulated cells compared with 15.0 ± 3.2 lipid bodies/cell in eotaxin-stimulated eosinophils (mean ± SD; n = 50 cells/group)].

Wet SEM

In both unstimulated and stimulated eosinophils, lipid bodies were clearly visible by wet SEM as highly contrasted, round organelles (**Fig. 2**). Lipid bodies appeared as bright white electron-dense structures (Fig. 2), as typically observed for lipid imaging of stained wet samples using BSEs (19). In addition, lipid bodies were quite distinct from eosinophil-specific granules, which could be seen as more individualized structures (**Fig. 3Ai**, arrows) compared with images seen by phase-contrast microscopy (Fig. 1Di). Computerized image processing (Fig. 3Aii) easily scored lipid body numbers and revealed a significant increase of them in eotaxin-stimulated eosinophils compared with unstimulated cells [8.5 ± 2.9 lipid bodies/cell in unstimulated cells compared with 20.4 ± 4.7 lipid bodies/cell in eotaxin-stimulated eosinophils (mean ± SD; n = 50 cells/group)], as also documented in this work by quantitative light microscopy.

Because of the higher resolution of SEM compared with light microscopy, a range of lipid body sizes was clearly observed, with an evident increase in the size of these organelles in activated cells (compare **Fig. 4A, B**). The wet SEM methodology also sharply demarcated the cell nucleus (Fig. 3Ai) and surface extensions (Fig. 4C). One frequent finding was the presence of lipid bodies close to or in contact with the perinuclear envelope (Fig. 4, arrows).

DISCUSSION

In contrast to traditional SEM, which is based on secondary electron detection and limits visualization to the cell surface, the wet SEM technique uses BSE detection, enabling the analysis of defined sample depth. In this work, we have demonstrated that wet SEM technology is particularly suitable for the rapid visualization and quantification of lipid bodies within human eosinophils. These multifunctional organelles are sites of eicosanoid formation in leukocytes and have received great attention because of their involvement with inflammatory diseases (4, 29, 30).

The sharp, highly contrasted lipid body imaging makes the wet SEM described here very useful for lipid body studies. This approach combines the advantages of light microscopy (i.e., rapid sample preparation; the complete processing requires ~1.5 h) and the resolution of electron microscopy, although it must be kept in mind that the resolution is lower compared with TEM. In addition, because the wet SEM approach facilitates the visualization of lipid accumulation, this technology is advantageous over fluorescent lipid probes by allowing lifelong detection in contrast to short-lived fluorescence. In fact, lipid body bleaching can be a limitation for routine analyses of these lipid-rich organelles by fluorescence microscopy, especially when quantitation is indispensable.

Another critical advantage of wet SEM is that it permits cell observation in a fully hydrated system. This is particularly important for lipid body studies, because these organelles are highly sensitive to dehydration processes, being dissolved when alcohol-based, routine staining methods are applied for light microscopy (15). Cell morphology also benefited from the wet SEM. Indeed, when we compared three different types of cell preparation (cytospin, agarose matrix, and wet SEM), better visualization of leukocyte morphology was achieved in cells kept hydrated within the capsules (Fig. 3A) or in the agarose matrix (Fig. 1A) compared with cytospin preparations (Fig. 1B).

Wet SEM has increasingly been applied to the study of different cells and tissues. In addition to facilitating lipid imaging, the technique is also useful, for instance, for rapid histopathological assessment (24, 31) and gold marker imaging (19, 32). Concerning lipid bodies, the greatest potential of wet SEM is its use for rapid and large-scale lipid body imaging and scoring within leukocytes and other cells under different conditions. Several technical considerations should be highlighted. First, a small volume of leukocyte suspension (15 μl/capsule) is sufficient for analysis. Second, the capsule membrane enables the rapid adherence of leukocytes (15 min). Third, leukocytes are rapidly fixed and stained (~1 h) when inside the capsules and can be imaged immediately after these procedures. Fourth, sealed capsules containing the samples are handy and can be stored at 4°C for further analysis (~1 week). Fifth, because lipid bodies exhibit high contrast, appearing as bright structures under wet SEM, computerized image processing (Fig. 3Aii) can easily score them, allowing the rapid analysis of a large number of cells. By light microscopy, lipid body enumeration is usually performed manually after osmium staining. Moreover, this staining may cause undesirable background in light microscopy (Fig. 1A), especially in cells in which the cytoplasm is packed with granules, and it may confound lipid body enumeration, in particular with small lipid bodies.

Finally, the wet SEM approach has the potential to be applied to lipid body analyses in a wide range of cells. Indeed, lipid bodies are present in most eukaryotic cells and have other important functional roles, being associated with membrane trafficking and other regulated processes (33–35). Wet SEM provides a strong imaging platform that can be used for multimodality lipid imaging, with special interest for the study of leukocyte lipid bodies in inflammation.

The authors thank Rebecca Stearns (Harvard School of Public Health, Boston, MA) and Ionita Ghiran (Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, MA) for technical support and Vered Behar (QuantomiX, Ltd.) for critical reading of the manuscript. This work was supported by National Institutes of Health Grants AI-20241 and AI-22571. R.C.N.M. was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil).

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