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Subcellular fractionation of human eosinophils: isolation of functional specific granules on isoosmotic density gradients

Josiane S. Neves[a], **Sandra A. C. Perez**[c], **Lisa A. Spencer**[a], **Rossana C. N. Melo**[a,b,d], and **Peter F. Weller**[a,*]

a *Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA, 02215, USA*

b *Department of Pathology, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA, 02215, USA*

c *Laboratory of Inflammation, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, RJ, 21045-900, Brazil*

d *Department of Biology, Federal University of Juiz de Fora Juiz de Fora, MG, 36036-330 Brazil*

Abstract

Subcellular fractionation has been an important tool in investigating human eosinophil structure and function, including localizing of cytokine/chemokines within granules, investigating granule protein translocation and intracellular transport during eosinophil secretion, and studying secretory mechanisms of granules. The resolution of organelles obtained by subcellular fractionation was improved considerably after the introduction of nonionic iodinated density-gradient metrizamide and Nycodenz media that, unlike sucrose, exhibit relatively low tonicity throughout the gradient. However, the structure and membrane preservation of isolated organelles were still compromised due to the lack of gradient isoosmolarity. This paper describes a detailed protocol of subcellular fractionation of nitrogen cavitated eosinophils on an isoosmotic iodinated density gradient (iodixanol − OptiPrep) and the isolation of well preserved and functional membrane-bound specific granules.

Keywords

granules; isoosmotic gradient; iodixanol; eosinophils

1. Introduction

Eosinophils, key cells in allergic reactions and in immune and inflammatory responses against parasitic helminths, are leukocytes that contain a dominant population of cytoplasmic specific granules ultrastructurally characterized by their crystalline core. Eosinophil specific granules are remarkable in their content of preformed basic proteins, such as eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN), preformed enzymes (eg, β-hexosaminidase) and a multiplicity of preformed cytokines/chemokines (Gleich, 2000; Rothenberg et al., 2006).

*Corresponding author: Peter F. Weller, E-mail: pweller@bidmc.harvard.edu, Beth Israel Deaconess Medical Center, Department of Medicine, vision of Allergy and Inflammation, 30 Brookline Avenue, CLS 943, Boston MA, 02215, tel:(617) 735 4110, fax: (617) 735 4115.

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Over the years, subcellular fractionation, performed by density gradient centrifugation, has been used as an important tool in the investigation of human eosinophil structure and function, including localizing of cytokine/chemokines within granules (Levi-Schaffer et al., 1995; Moqbel et al., 1995; Levi-Schaffer et al., 1996; Lacy et al., 1998; Lacy et al., 1999; Woerly et al., 2002; Spencer et al., 2008) and studying granule protein translocation and intracellular transport during eosinophil secretion (Lacy et al., 1998; Lacy et al., 1999; Karawajczyk et al., 2000; Logan et al., 2002; Logan et al., 2006; Spencer et al., 2006; Spencer et al., 2008).

Sucrose gradients have been used for the characterization (West et al., 1975; Lewis et al., 1978) and for the understanding of the selective mobilization of eosinophil granule proteins (Karawajczyk et al., 2000). However, limitations of sucrose-based gradients and their high osmotic pressure/density ratio that can cause reversible or irreversible shrinkage to organelles and thereby reduce density differences between certain types of organelles, affecting the resolution of the gradient (Castle, 2003; Castle, 2004). More recently, there has been renewed interest in other iodinated media like metrizamide and Nycodenz for use in separation by isopycnic banding in subcellular fractionation. Nycodenz, much less osmotically active and less viscous than sucrose, can generally resolve organelles and vesicles, and thus has been widely used for human eosinophil subcellular fractionations (Levi-Schaffer et al., 1995; Moqbel et al., 1995; Levi-Schaffer et al., 1996; Lacy et al., 1998; Lacy et al., 1999; Logan et al., 2002; Woerly et al., 2002; Logan et al., 2006). However, despite presenting reduced osmolarity and viscosity compared to sucrose, Nycodenz and metrizamide still exert a significant osmotic pressure in higher densities (Castle, 2004). For instance, both of them are only isoosmotic with mammalian fluids (290–300 mOsm) at densities below ~ 1.2 g/mL which is above that of most organelles (Castle, 2004).

The latest addition to the range of iodinated density gradient media is iodixanol (OptiPrep), which enables preparation of isoosmotic solutions over the full range of organelle densities, making this medium ideal for the isolation of organelles for functional studies. The physical properties of iodixanol have been described previously (Ford et al., 1994). Briefly, iodixanol is essentially a dimer of Nycodenz. It is available commercially as a 60% (w/v) solution in water whose osmolality is approximately 260 mOsm. Using an osmotic balancer it can form isoosmotic gradients over a wide range of densities (up to 1.32 g/mL).

Here we describe in detail an optimized process of subcellular fractionation of nitrogen cavitated human eosinophils using an iodixanol isoosmotic density gradient. This approach enables isolation of functional well-preserved membrane-bound eosinophil specific granules and was recently used by our group to document the secretory properties of these granules as cell-free organelles (Neves et al., 2008).

2. Methods

2.1. Isolation of human eosinophils

Eosinophils were isolated as described (Neves et al., 2008). Eosinophils were obtained from the blood of healthy and atopic donors. Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. Blood was mixed with neutral sodium citrate (100 mM, pH 7.4) and 6% dextran-saline (Baxter, Deerfield, IL, USA) in a ratio of 4:1:1 for 30–45 min to allow erythrocyte sedimentation. The leukocyte-enriched plasma was harvested, layered onto Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) and centrifuged at 250*g* for 20 min with a starting temperature of 25°C reaching 4°C by the end of centrifugation. Except for blood collection and dextran sedimentation of erythrocytes, the procedure was carried out at 4°C. The granulocyte-enriched cell pellets were collected, washed at 4°C with calcium-, magnesium-free HBSS and incubated with anti-CD2, -CD16, -CD14, -CD19, glycophorin and

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-CD56 immunomagnetic beads as per manufacturer's instructions (StemSep eosinophil enrichment cocktail, Stem-Cell Technologies, Vancouver, Canada). Eosinophils were negatively selected using the MACS system (Miltenyl Biotec, Aubum, CA, USA). Erythrocyte hypotonic lysis was not performed. Contaminating RBCs were eliminated by anti-glycophorin antibodies (Abs) included in the negative selection eosinophil enrichment antibody (Ab) cocktail. The viability and purity of freshly isolated eosinophils were >99% as analyzed by trypan blue exclusion and HEMA3 staining (Fisher Scientific Co., Pittsburgh, PA, USA), respectively. For subcellular fractionations, a number of eosinophils not less than 10×10^6 and not more than 35×10^6 was used in order to obtain a reasonable amount of eosinophil specific granules and to prevent overloading the gradient, respectively.

2.2. Disruption of eosinophils by nitrogen cavitation

Isolated eosinophils ($10–35 \times 10^6$) were resuspended in 1 mL of disruption buffer (0.25 M sucrose, 1 mM EGTA, 10 mM Hepes – pH 7.4) supplemented with 100 μg/mL phenylmethylsulfonylfluoride (PMSF), 2 mM $MgCl_2$, 1mM ATP and 5 μg/mL each of pepstatin, N-alpha-p-tosyl-L-arginine methyl ester hydrochloride (TAME), aprotinin, 1,4-dithio-DL-threitol (DTT) and leupeptin, all of them added just before use. Cells were pressurized under nitrogen for 10 min at 600 psi in a nitrogen bomb (Parr Instrument, Moline, IL, USA) at 4°C. The cavitate was then collected drop by drop very slowly and gently. Nuclei and intact cells were pelleted by centrifugation at 200g, 4°C for 10 min. The postnuclear supernatant (Sup 1) was carefully decanted, kept at 4°C and the pellet (P1) was resuspended in another 1 mL of protease inhibitor supplemented disruption buffer and submitted to a second nitrogen cavitation. The second cavitate was centrifuged (200 g, 4°C, 10 min) and the second postunuclear supernatant (Sup 2) was joined to Sup1. Sup 1 + Sup 2 were submitted to one more centrifugation (200g, 4°C, 10 min) and further analyzed by light microscopy to ensure the absence of intact cells or nuclei. All cell and granule manipulations were performed very gently with plastic Pasteur pipettes at 4°C in order to minimize damage or activation.

2.3. Density centrifugation of iodixanol gradient

2.3.1. Preparation of iodixanol—Iodixanol solution (45%) was prepared from a ready made mixture of iodixanol/water 60% (w/v) commercially available under the name of OptiPrep (OptiPrep, Axis-Shield, Oslo, Sweden, density: 1.32 ± 0.001g/mL; osmolality: 170 ± 15 mOsm).

Iodixanol solution (45%) was prepared after two subsequent dilution steps as follow. First, to prepare a 50% (w/v) solution, 5 vol of Iodixanol 60% was mixed with 1 vol of the diluent (0.25 M sucrose, 6mM EGTA, 60 mM Hepes, pH=7.4). To reach a 45% (w/v) solution, 9 vol of iodixanol 50% (w/v) was mixed with 1 vol of the disruption buffer (0.25M sucrose, 1 mM EGTA, 10 mM Hepes – pH 7.4) with no protease inhibitor addition. According to the OptiPrep manufacturer, the density of the iodixanol 45% (w/v) gradient solution prepared as described above is between 1.243 to 1.252 g/mL. The density of iodixanol 45% (w/v) in our conditions was 1.248 g/ml. Six mL aliquots of iodixanol 45% (w/v) and disruption buffer were frozen at −20°C until use.

2.3.2. Continuous iodixanol gradient preparation—The continuous iodixanol gradient was made using a standard two chamber gradient maker connected to a peristaltic pump set to ~ 180 μL/min. A cushion of 0.5 mL iodixanol 45% was layered on the bottom of the tube (open top polyclear centrifuge tubes − 14 × 89 mm – Seton Scientific, Los Gatos, CA, USA). The high-density medium (iodixanol 45% - 5 mL) was placed in the mixing chamber (the one connected to the delivery tube) and the low-density medium (the same composition as the disrupt buffer − 0.25 M sucrose, 1 mM EGTA, 10 mM Hepes – pH 7.4 with no protease inhibitor supplementation – 4mL) was placed in the other chamber. The tip of the delivery tube needs

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to be placed against the wall of the centrifuge tube close to its top. The incoming gradient is allowed to flow in a steady stream down the wall of the tube (Fig. 1A). The gradient was prepared freshly on the day of the experiment and kept at 4°C until use.

2.3.3. Gradient loading and centrifugation—Two mL of the postnuclear supernatant (Sup1+Sup2) described in 2.2 was applied slowly through a plastic Pasteur pipette on top of the pre-cooled gradient. The loaded gradient was ultracentrifuged at 100,000 g for 1h at 4°C. After ultracentrifugation, the eosinophil specific granule band can be visualized at denser gradient fractions as indicated in Fig. 1B.

2.4 Fractionation

Fractions were collected at 4°C in 1.5 mL test tubes by aspiration from the bottom, through a capillary tube attached to a polyethylene tube connected to a peristaltic pump and a fraction collector (total of 20 fractions – ~500 μl each, ~12 drops). The granule-enriched fractions are normally fractions number 5 and 6 (from the bottom to the top). Collection from the top to the bottom should be avoided in order to prevent possible contamination with secretory vesicles and plasma membrane fragments. The fractions density (Fig. 2A) was determined by the use of a digital densitometer (DMA 35, Mettler/Paar, Graz, Austria). According to iodixanol (OptiPrep) specification sheet, the osmolarity of the iodixanol solutions in the referred fraction densities is in the range 295–310 mOsm.

2.5. Fraction identification

EPO activity and lysosome-associated membrane protein-2 (LAMP-2) were used as markers for specific granules- and secretory vesicles-containing fractions. Secretory vesicle-containing fractions were additionally identified by localization of vesicle-associated membrane protein-2 (VAMP-2). Major histocompatibility complex class I (MHC class I) and lactate dehydrogenase (LDH) were used as markers for plasma membrane- and cytoplasm-containing fractions, respectively. EPO activity was measured by a colorimetric assay as described previously (Kroegel et al., 1989) with some modifications. Briefly, 20 μL 5x diluted subcellular fractions were incubated with 100 μL substrate solution (50 mM Tris-HCl, 1 mM H_2O_2, 2 mM O-phenylenediamine dihydrochloride (OPD) and 0.1% Triton X-100, pH=8). The reaction was stopped after 30 minutes of incubation by the addition of 100 μL 4M H_2SO_4. The plate was read at 492 nm.

LAMP-2, VAMP-2, MHC class I and LDH were characterized on granule fractions by Western blotting. Briefly, 30 μl of each fraction was mixed with reducing buffer (LAMP-2, VAMP-2 and LDH) or non-reducing buffer (MHC class I) and boiled for 5 min. Samples were loaded on 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and run using MOPS (LAMP-2, LDH and MHC class I) and MES (VAMP-2) as running buffers, under denaturing conditions. Gels were transferred to nitrocellulose membranes (Thermo Scientific, Waltham, MA, USA), blocked overnight with 5% milk, and probed with rabbit anti-LDH monoclonal Ab (1:3000, Abcam, Cambridge, MA, USA, clone EP1566Y), rabbit anti-VAMP-2 polyclonal Ab (1:2000, Assay Designs, Ann Arbor, MI, USA), mouse anti-LAMP-2 monoclonal Ab (1:200, BD Pharmingen, San Diego, CA, USA, clone H4B4) and mouse anti-MHC class I monoclonal Ab (1:250, Abcam, clone W6/32). Anti-rabbit and anti-mouse Abs conjugated to horseradish peroxidase (HRP) (1:15000, Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary Abs. Membranes were developed with West Pico chemiluminescence kits (Thermo Scientific).

2.6. Eosinophil specific granule isolation and characterization

Granule fractions (usually fractions #5 and #6) were pooled and split into aliquots depending on experimental groups. Each sample was washed well with calcium- magnesium-free HBSS

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(2 mL). Granules were pelleted by centrifugation using a swinging-bucket rotor (to ensure that the granule pellet will be located on the bottom of the tube) at 2,500 g × for 10 min and processed for flow cytometry or transmission electron microscopy (TEM) as follows.

2.7. Flow cytometry of eosinophil granules

Isolated granules were incubated either with primary fluorescein isothiocyanate (FITC)-conjugated Ab (45 min) or primary (1 h) and then FITC-conjugated secondary (15 min) Abs on ice in the absence of granule fixation. After staining, granules were fixed in buffer containing 2% paraformaldehyde (PFO) without methanol (Electron Microscopy Sciences, Fort Washington, PA, USA) for 5 min. For intragranular staining, isolated granules were fixed for 5 min in 2% PFO and permeabilized for 5 min on ice with 0.1% saponin (Sigma, Saint Louis, MO, USA) before incubation with primary (1h) and secondary (15 min) Abs in the continued presence of saponin. After staining, samples were stored in saponin-free buffer. Control or non-immune Abs were included for all. Analyses were performed on a FACScan with CELLQUEST software (BD Biosciences, San Jose, CA, USA). For non-permeabilized granules, mouse anti-human CD63 (LAMP-3) monoclonal Ab (clone H5C6, 20 μg/mL; BD Pharmingen) was used in parallel with respective isotype control irrelevant IgGs. An anti-mouse FITC-conjugated Ab was used as a secondary Ab (1:100, Jackson ImmunoResearch). Mouse anti-human CCR3 (clone 61828.11, 5 μg/mL, R&D) and mouse anti-human MHC class I (HLA-ABC, clone G46–2.6; 14 μg/mL; BD Pharmingen) FITC-conjugated monoclonal Abs, each directed against extracellular domains, were used in parallel with respective FITC-conjugated IgG control monoclonal Abs. On permeabilized granules, mouse anti-human major basic protein (MBP) monoclonal Ab (5 μg/mL, clone AHE-2, BD Pharmingen) and an isotype monoclonal Ab were used; and the secondary Ab was a FITC-conjugated goat anti-mouse Ab (1:100; Jackson ImmunoResearch).

2.8. Granule preparation and analysis by TEM

Granule samples (n=15) in agar from different experiments were prepared and analyzed by TEM as before (Neves et al., 2008). Briefly, granule samples were fixed in a mixture of freshly prepared aldehydes (1% PFO and 1.25% glutaraldehyde) in 1 M sodium cacodylate buffer, pH 7.4, for 30 min, at room temperature, washed in the same buffer and centrifuged at 1500 g for 1 min. They were then resuspended in molten 2% agar in 1 M sodium cacodylate buffer, pH 7.4, and quickly recentrifuged. Resultant agar samples were kept in the same buffer at 4°C for further processing. Samples were postfixed in 1% osmium tetroxide in Sym-Collidine buffer, pH 7.4, for 2 h at room temperature. After washing with sodium maleate buffer, pH 5.2, they were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0, for 2 h at room temperature and washed in the same buffer as before prior to dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA). After polymerization at 60°C for 16 h, thin sections were cut using a diamond knife on a Leika ultramicrotome (Leica, Bannockburn, IL, USA). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM10; Philips, Eindhoven, the Netherlands) at 60 KV. At least, 450 electron micrographs randomly taken at 5,000x – 30,000x were analyzed.

2.9. Acridine orange (AO) granule-loading

AO labels acidic lysosomal eosinophil granules (Stankiewicz et al., 1996). Cells and granules were loaded with AO (Sigma) 3 μM (10 min, 4°C) in Ca^{++}/Mg^{++}-free HBSS. Granules were spread on poly-L-lysine coated slides (5 μl/slide) and coverslipped. Fluorescence images were acquired using a Hamamatsu Orca AG cooled monochrome CCD camera (Bridgewater, NJ, USA) coupled to a BX-62 Olympus microscope (Olympus, Center Valley, PA, USA) using a

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UplanApo objective (60 × 1.35). The microscope, Z-motor drive and camera were controlled by IVision 4.0 (BioVision Technologies, Exton, PA, USA).

3. Results

3.1. Detection of specific markers in the respective subcellular fractions

As illustrated in Fig. 2A, highest levels of EPO activity were identified on heavier granule-enriched fractions 5 and 6 (mean density = 1.196 g/ml). Considerable, but much lower, EPO activity was also identified in more buoyant light fractions 16 and 17, probably corresponding to small secretory vesicle EPO content. By Western, LAMP-2, a marker for eosinophil granules and secretory vesicles (Persson et al., 2002), was identified on granule- (#5 and #6) and small secretory vesicle-enriched fractions (#17 and #18; density = 1.030 g/ml) (Fig. 2Bi). Additionally, as showed in Fig. 2Bii, vesicle-containing fractions showed immunoreactivity for VAMP-2, a marker for secretory vesicles, but not for granules, as previously described (Lacy et al., 2001). The cytosol (density = 1.030 g/ml) (Fig. 2Biii) and the plasma membrane fractions (density range = 1.080–1.030 g/ml) (Fig. 2Biv) were also detected by Western blot after probing with anti-LDH and anti-MHC class I Abs, respectively.

3.2. Flow cytometry of eosinophil isolated granules

Granules were characterized by flow cytometry using the forward (FSC) and side scatter (SSC) settings in logarithmic scales as demonstrated in Fig. 3A. To assess if the gated region corresponded to eosinophil granules we performed a series of labeling for granule marker proteins. As illustrated in Fig. 3B, MBP/SSC dot plot with the gate marked (G1) contained an increased MBP positive granule population after labeling with an anti-MBP monoclonal Ab under permeabilized conditions and in comparison with the control Ab. Under non-permeabilized conditions, this granule population was positive after staining with CD63 (also known as LAMP-3) (Fig. 3C), previously localized to eosinophil granules (Mahmudi-Azer et al., 2002). In addition, granules isolated by subcellular fractionation in our conditions did not express contaminating plasma membrane or endossomal MHC class I protein as assessed by flow cytometry after staining with anti-MHC I monoclonal Ab (Fig. 3D). Granules, also showed positivity for CCR3, the G protein coupled-receptor for eotaxin, as illustrated in Fig. 3E (Neves et al., 2008).

3.3. Acridine orange granule staining

Eosinophil granules can be visualized under light and fluorescence microscopy as illustrated in Fig. 4. By light microscopy, granules can be observed either intracellularly (Fig. 4Ai) or extracellularly, in clusters (Fig. 4Bi) or isolated (Fig. 4Ci). After acridine orange staining, a marker for intracellular acidic compartments, these organelles can be visualized clearly under fluorescence microscopy within intact eosinophils (Fig. 4Aii) and after isolation (Fig. 4Bii and 4Cii), suggesting that the acidic properties of eosinophil granules were preserved during the purification process.

3.4. TEM of eosinophil isolated granules

The extensive analysis of granules by TEM revealed that granule-enriched fractions were free of other organelles. Moreover, purified granules exhibited well preserved surrounding bilayer membranes as illustrated in Fig. 5A and B (arrowheads and higher magnified in Ai and Bi).

4. Discussion

Here we report a method to isolate well-preserved and functional eosinophil granules combining nitrogen cavitation and the use of an isoosmotic iodixanol gradient. The approach

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described here significantly avoids the shrinkage and damage of granules during subcellular fractionation, preserving their structure, properties and functionality. After eosinophil nitrogen cavitation and gradient centrifugation, granule-enriched fractions were identified by their content of EPO activity and LAMP-2 immunoreactivity. We characterized and confirmed the purity of our isolated granules by electron microscopy, Western blot and flow cytometry, which indicated that the granule fractions were free of other organelles and absent of membrane MHC class I and cytoplasmic protein contamination. The flowcytometric analysis of isolated granules also revealed granule immunoreactivity for MBP and CD63 and the expression of the CCR3 receptor for eotaxin on granule membranes, as previously described (Neves et al., 2008). Furthermore, granules isolated under our conditions preserved their acidic properties as demonstrated by AO staining and displayed intact surrounding bilayer membranes which is essential for subsequent studies on granule functionality.

Sucrose and later Nycodenz have been widely used as separating media in eosinophil subcellular fractionations to characterize eosinophil granule protein content and release (West et al., 1975; Lewis et al., 1978; Levi-Schaffer et al., 1995; Moqbel et al., 1995; Levi-Schaffer et al., 1996; Lacy et al., 1998; Lacy et al., 1999; Karawajczyk et al., 2000; Logan et al., 2002; Woerly et al., 2002; Logan et al., 2006). One of the first reports indicating the isolation and partial characterization of human eosinophil granules describes the existence of an eosinophil granule band with a mean density around 1.24 g/mL after isopycnic ultracentrifugation in a sucrose gradient (West et al., 1975). A more recent work (Levi-Schaffer et al., 1996), using a Nycodenz gradient (0–45%), suggests an eosinophil granule band around 1.2 g/mL. Solutions of Nycodenz can only be made isoosmotic below 1.16 g/mL (Castle, 2004). Thus, Nycodenz at 1.2 g/mL can be significantly hyperosmotic. In early stages of this work, attempts to isolate vesicles and granules by subcellular fractionation using Nycodenz gradient and their analysis by TEM had failed. We were unable to isolate eosinophil vesicles probably due their tendency to shrink under hyperosmotic conditions. Regarding granules, the results were inconsistent with granules presenting unpreserved morphology in the majority of the preparations (data not shown). An advantage of iodixanol over previously iodinated density gradient media is that its aqueous solutions are isoosmotic up to a density of 1.32 g/mL, enabling preparation of isoosmotic solutions over the range of eosinophil granule density (granule band mean density in our iodixanol gradient = 1.196 g/ml) and making this media more ideal for isolation of organelles for functional studies. Iodixanol gradients have been used before for subcellular studies in different cell types like mouse hepatic cells and epithelial cells (Graham et al., 1994; Li et al., 2008). In previous papers published by our group, we described for the first time the use of the isoosmotic iodixanol gradient for eosinophil subcellular fractionation in eosinophil vesicles isolation (Melo et al., 2005), in the investigation of selective cytokine intracellular transport during eosinophil secretion (Spencer et al., 2006) and in the study of granule content and release of Th1 cytokines (Spencer et al., 2008). Recently, we described the use of iodixanol for isolation of well preserved, membrane-bound granules capable of functioning extracellularly as cell independent secretory organelles (Neves et al., 2008). Here, we are providing the detailed protocol. Summarizing, subcellular fractionation of nitrogen cavitated eosinophils on isoosmotic iodixanol gradients is a simple and very reproducible technique that permits isolation of eosinophil granules under isoosmotic conditions enabling more ideal conditions for the preservation of the functional characteristics of this organelle.

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Abbreviations

Ab
antibody

Abs
antibodies

AO
acridine orange

DTT
dithio-DL-threitol

ECP
eosinophil cationic protein

EDN
eosinophil derived neurotoxin

EPO
eosinophil peroxidase

FITC
fluorescein isothiocyanate

FSC
forward scatter

HRP
horseradish peroxidase

LAMP
lysosome-associated membrane protein

LDH
lactate dehydrogenase

MHC
major histocompatibility complex

MBP
major basic protein

OD
optical density

OPD
O-phenylenediamine dihydrochloride

PMSF
phenylmethylsulfonylfluoride

PM
plasma membrane

SSC
side scatter

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TAME

N-alpha-p-tosyl-L-arginine methyl ester hydrocloride

TEM

transmission electron microscopy

VAMP

vesicle-associated membrane protein

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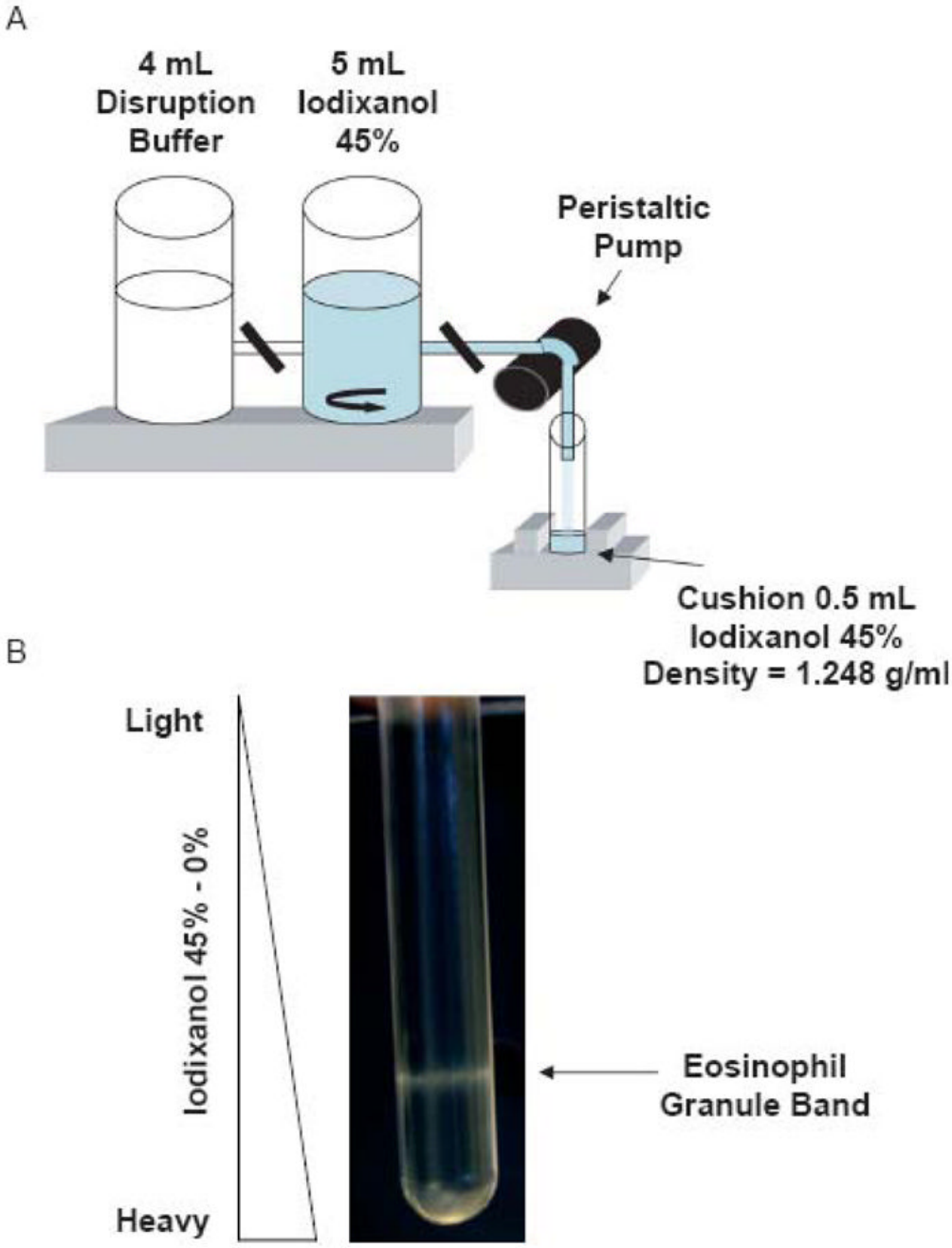

Fig. 1.

A. Continuous gradient-forming device showing the back reservoir containing the lower-density solution (4 mL disruption buffer) connected to the mixing chamber containing the higher-density solution (5 mL iodixanol 45%) via a channel with a stopcock. A delivery tube leads from the mixing chamber to a peristaltic pump and then to the top wall of the centrifuge tube containing a cushion of 0.5 mL iodixanol 45%. B. After eosinophil disruption, 2 mL postnuclear supernatants were loaded onto an iodixanol gradient. After centrifugation at 100,000 g for 1h, the gradient shows an eosinophil granule band as indicated by the arrow.

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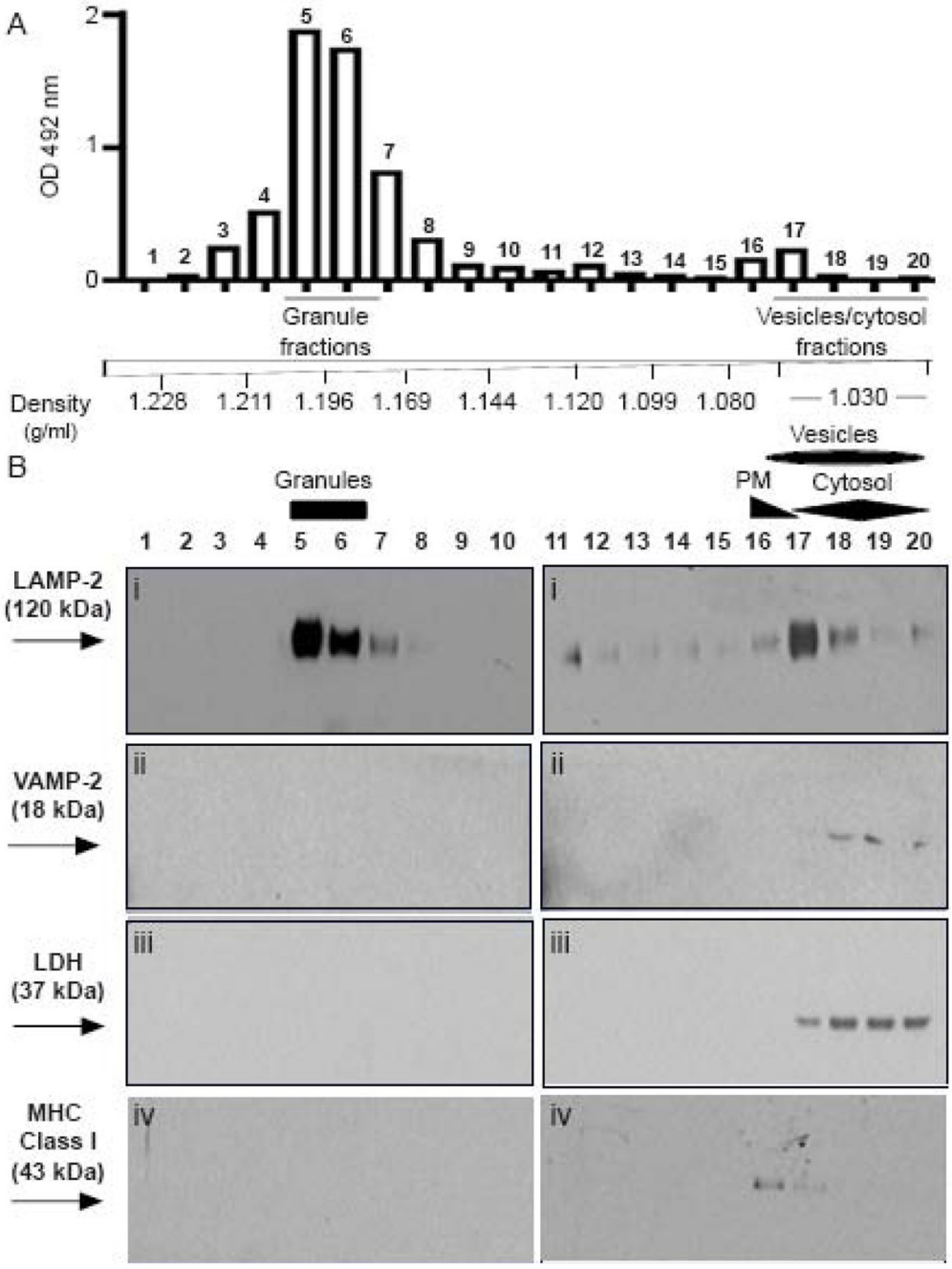

Fig. 2.

A. After density gradient fractionation, the highest eosinophil peroxidase (EPO) enzymatic activity (optical density (OD) 492 nm) was observed in denser granule-containing fractions and less dense secretory vesicles. B. Granule, vesicle, cytoplasm and plasma membrane markers. Bi. Lysosome-associated membrane protein-2 (LAMP-2) immunoreactivity identifies granule and vesicle localization within subcellular fractions. Bii. Vesicle-associated membrane protein-2 (VAMP-2) reactivity identifying vesicle-containing fractions. Biii. Lactate dehydrogenase (LDH) and Biv. major histocompatibility complex class I (MHC class I) as markers for cytoplasm- and plasma membrane-containing fractions, respectively. The gradient was fractionated using a peristaltic pump from the bottom to the top of the tube and

proteins in each fraction were then analyzed. Eosinophil subcellular fractions were separated on the basis of their density range (1.228-1.030 g/ml) on iodixanol gradient. PM = plasma membrane

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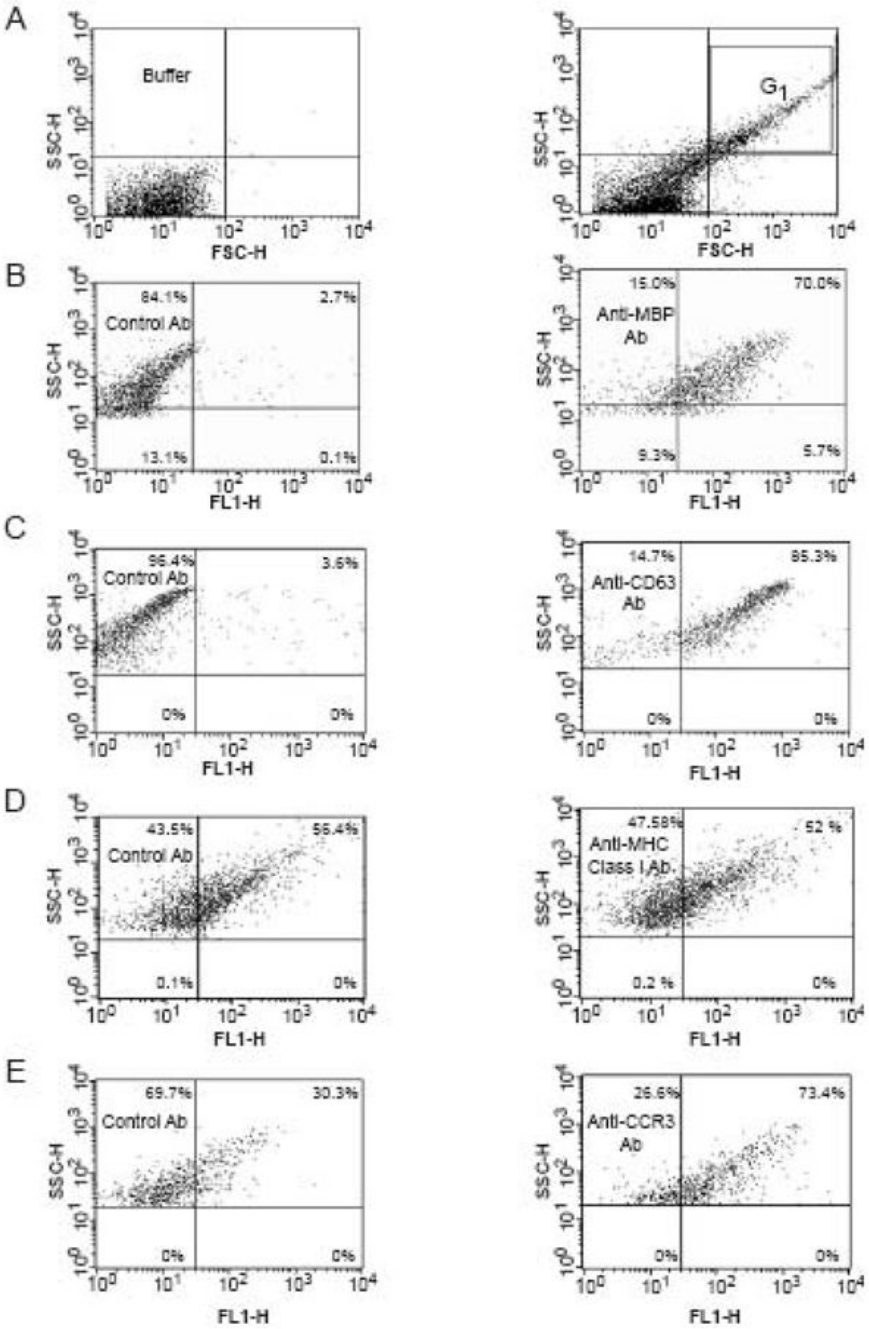

Fig. 3.

A. Granules were analyzed by flow cytometry using the forward (FSC) and side scatter (SSC) settings in logarithmic scales. B. Isolated membrane-permeabilized granules were positive for major basic protein (MBP) (left panel) after staining with anti-MBP monoclonal Ab and compared to control Ab (right panel). C. Non-permeabilized granules express immunoreactivity for CD63 (also know as LAMP-3) when compared to control Ab (right panel). C. Granules isolated by subcellular fractionation do not contain contaminating plasma membrane or endosomal major histocompatibility complex (MHC) class I protein after staining with anti MHC class I monoclonal Ab (right panel) and compared to control Ab (right panel).

D. Non-membrane permeabilized granules expressed “extracellular” domains of the CCR3 receptor for eotaxin (left panel) when compared to control Ab (right panel), $n \geq 3$.

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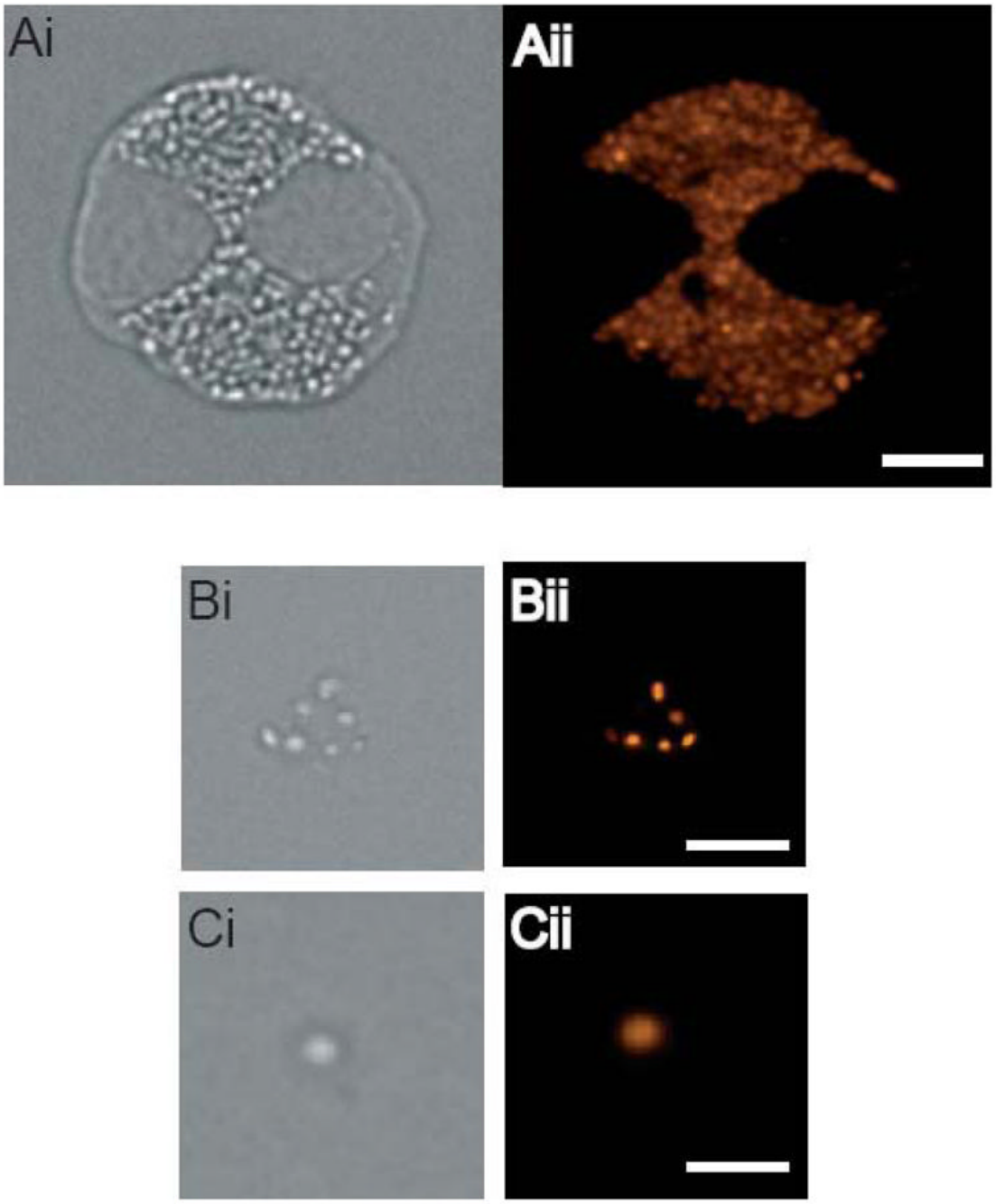

Fig. 4.

Eosinophil granules observed either intracellularly (Ai) or following subcellular fractionation and isolation, in clusters (Bi) or singularly (Ci). By fluorescence microscopy and after acridine orange staining, eosinophil granules can be visualized within intact eosinophils (Aii) and after isolation (Bii and Ciii). Scale bar: 3 μm (Ai and Aii), 3.6 μm (Bi and Bii), 1.8 μm (Ci and Cii).

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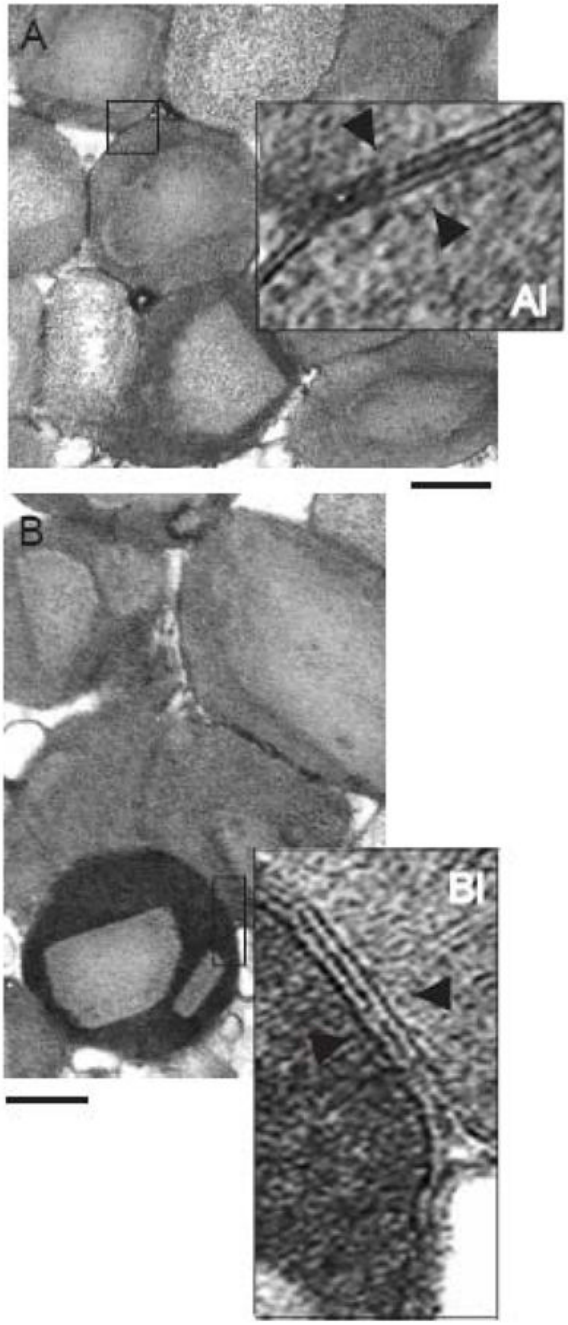

Fig. 5.

Transmission electron microscopy identified granules with well-preserved surrounding membranes. The granule membranes trilaminar structure (arrowheads) is shown in higher magnification in the boxed areas. Scale bar: 276 nm (A) and 50 nm (higher magnification Ai), 233 nm (B) and 50 nm (higher magnification Bi).