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A Gel-Based Dual Antibody Capture and Detection Method for Assaying of Extracellular Cytokine Secretion

EliCell

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Summary

A distinguishing feature of eosinophils is their ability to rapidly release preformed cytokines from intracellular pools. Cytokines are delivered to the cell surface from granule stores by transport vesicles and are released in small packets at discrete locations along the cell surface through a process termed "piecemeal" degranulation. The study of this process has been hindered by lack of an assay sensitive enough to register minute protein concentrations and the inability to visualize morphology of cytokine secreting cells. These hindrances have necessitated our development of the EliCell assay, an agarose-based dual cytokine capture and detection system through which cytokine secretion and cellular morphology may be analyzed in concert. Cells are embedded within capture antibody-containing agarose and stimulated under conditions of interest. Extracellularly released cytokine is captured within the matrix at the point of release from the cell and can be labeled with a fluorochrome-conjugated antibody. Cytokine release and cellular morphology are visualized in parallel by phase contrast and fluorescence microscopy, respectively.

Key Words: EliCell; agarose matrix; eosinophil; cytokine; piecemeal degranulation; vesicular transport; secretion.

1. Introduction

Eosinophils have long been noted for their content of cationic granule proteins, the deposition of which leads to tissue damage and cellular dysfunction *(1,2)*. A more recently noted characteristic of eosinophils is their internal stores of preformed cytokines and chemokines with a wide range of biological functions *(3)*. In most other cells (i.e., T-cells), the release of these cytokines depends

upon *de novo* synthesis in response to a particular stimulus. The ability of eosinophils to release these potent immunomodulators in the absence of *de novo* synthesis highlights the potential of these cells to play key roles in rapidly affecting the initiation or course of an allergic response.

Most, if not all, of the cytokines found preformed within eosinophils have been localized to specific granules *(4–9)*. Strong evidence provided mainly through electron microscopic analyses suggests that the mobilization of these factors from granule stores and subsequent extracellular release follows a process termed "piecemeal" degranulation (PMD), where small cytokine-containing vesicles traffic from granules to the plasma membrane. This process results in small packets of material being released at discrete locations along the cell surface. Although exocytosis of entire granule contents may occur under rare conditions, the more measured, specific process of PMD predominates *(4,10–12)*. Despite colocalization of numerous factors within specific granules and the common use of PMD as a method of exiting the cell, cytokines and chemokines are likely released individually in a tightly regulated, stimulus-dependent manner.

An understanding of molecular mechanisms responsible for this high level of specificity has been hindered by the lack of appropriate assays to detect low levels of stimulus-dependent cytokine release. Enzyme-linked immunosorbent assay (ELISA) methods relying on measurement of cytokine/chemokine levels in culture supernatants have been successful in the detection of some eosinophil-derived cytokines, particularly when nonphysiological stimuli and long incubation times are employed *(13–15)*. However, the progressive nature of release by PMD in response to more physiological stimuli cannot be fully appreciated because the released quantities are below the sensitivity of the assay. An additional obstacle is the potential of eosinophils to recapture or sequester released product with specific cytokine receptors at the cell surface, giving the false impression of lack of output *(16)*.

Enzyme-linked immunospot (ELISPOT) techniques avoid many of these disadvantages and provide the potential to detect minute quantities of product at the individual cell level. However, this approach provides little information concerning morphology, activation status, or even the viability of individual cytokine-producing cells. This can be especially important when considering eosinophil populations which, unlike most other cell types, do not require stimulus-induced de novo synthesis of the cytokine in question. Therefore, a damaged or permeabilized cell may appear as a positive spot because of the artificial release of its preformed contents or availability of intracellular stores to detecting antibodies.

In light of the unique difficulties introduced in the study of eosinophil PMD, we have developed a new approach to visualize the release of specific cytokine and chemokine products. Based upon the dual antibody capture and detection

system of ELISPOT, the EliCell assay uses an avidin-conjugated agarose matrix to bind biotinylated cytokine-specific capture antibody. Cells are embedded within (rather than atop) this matrix and stimulated under conditions of interest. Released cytokine is captured within the matrix at the point of release from the cell and can be detected by a fluorochrome-labeled detection antibody. Viable cells remain embedded within the agarose substrate throughout the procedure and can be visualized under high magnification by phase contrast for morphological analysis in parallel with detection of released product by fluorescence microscopy *(17,18)*.

This approach provides substantial advantages, namely the ability to microscopically observe the cytokine-secreting cell in parallel with detection of the secreted product, providing information on viability and potential polarization of factor release. In addition, the procedure may be easily modified to allow for simultaneous detection of multiple cytokines, observance of surface markers in conjunction with cytokine release, detection of intracellular products, and other cytochemical analyses.

2. Materials

2.1. Agarose Activation

1. Low-melting point agarose (mp 65.5°C, gelling point 24°C; Promega, Madison, WI; cat. no. V2111).
2. 10 mM NaIO$_4$ dissolved in 100 mM sodium acetate buffer, pH 5.5.
   Store at 4°C and protect from light.
3. 70°C Water bath.

2.2. Coupling of Activated Agarose to Avidin

2.2.1. Coupling of Streptavidin to Agarose

1. Streptavidin-hydrazide (Pierce Chemical, Rockford, IL; cat. no. 21120).

2.2.2. Coupling of NeutrAvidin to Agarose

1. NeutrAvidin biotin-binding protein (Pierce; cat. no. 31000).
2. Quenching buffer (pH 7.4; may be purchased, along with sodium cyanoborohyde in **step 3**, in the AminoLink Plus Immobilization Kit [Pierce, cat. no. 44894]): 1 M Tris-HCl; 0.05% NaN$_3$.
3. Sodium cyanoborohydride* (32 mg; Pierce; cat. no. 44892) dissolved in 5 mL of 10 mM NaOH.
   Because of the high toxicity of CNBH$_3$, this step should be performed in a fume hood.
4. ImmunoPure HABA (Pierce; cat. no. 28010).

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* Reagents may be purchased together in AminoLink Plus Immobilization Kit (Pierce, cat. no. 44894.

2.3. EliCell Assay

1. 37°C Water bath.
2. 10X RPMI-1640 (Sigma-Aldrich).
3. 1X RPMI 1640 (Sigma-Aldrich) supplemented 0.1% ovalbumin.
4. Biotinylated capture antibody (0.1 mg/mL diluted in RPMI + 0.1% ovalbumin).
5. Ultra-microtips with elongated tip (USA Scientific, Ocala, FL; cat. no. 1111-4000).
6. Microscope slides and cover slips (22 × 50 mm).
7. CoverWell perfusion chambers (Grace Bio-Labs, Bend, OR, cat. no. PC1L-0.5).
8. Hanks Buffered Salt Solution without calcium chloride and magnesium chloride (hereafter HBSS –/–).
9. Paraformaldehyde (diluted to 2% in HBSS –/–) (Electron microscopy Sciences, Ft. Washington, PA; cat. no. 15710). Dilutions should be made in fume hood and fresh dilutions of PFO should be used in each experiment. Protect from light.
10. Detection antibody pre-labeled with fluorochrome (*see* **Note 1**).
11. Aqua Poly/Mount (Polysciences, Inc., Warrington, PA; cat. no. 18606).
12. Acridine orange: ethidium bromide solution (recipe for solution adapted from Becton Dickinson *Immunocytometry Systems Cytometry Source Book*). Prepare 100X stock: 50 mg of ethidium bromide; 15 mg of acridine orange; dissolve in 1 mL of 95% ethanol. Add 49 mL of dI water. Mix well, divide into 1-mL aliquots and freeze. 1X working solution (prepare fresh): Dilute 100X stock with PBS and mix well. Store at 4°C for up to 1 mo protected from light.
13. Fast Green (Sigma).
14. Hematoxylin (Sigma).
15. Hema 3 Staining kit (Pierce).
16. AX-70 Provis Olympus with FITC and TRITC filter sets (HBO burner). Objectives used: 40X 1.00 Ph3 UplanApo and 100x 1.35 Ph3 UplanApo

3. Methods

3.1. Activation of Agarose

Incorporation of capture antibody into the support matrix requires prior activation of the agarose to generate functional aldehydes. Oxidation of *cis*-vicinal hydroxyl groups of the agarose by sodium metaperiodate is detailed below and in **Fig. 1A**.

1. Weigh 0.125 g of low-melting point agarose into a 250-mL Erlenmeyer flask (to increase surface area) and dilute to 2.5% by adding 5 mL sterile dI water
2. Mix well, but avoid swirling to prevent agarose binding to flask wall.
3. Solubilize agarose in 70°C water bath for 15 min with gentle agitation.
4. Solidify agarose at 4°C for 20 min.
5. Cover surface of solidified agarose with 5 mL of periodate solution (10 mM NaIO$_4$ in 100 mM sodium acetate buffer, pH 5.5) to activate the agarose, generating reactive aldehyde groups.
6. Protect from light and store overnight at 4°C.
7. Discard periodate solution.
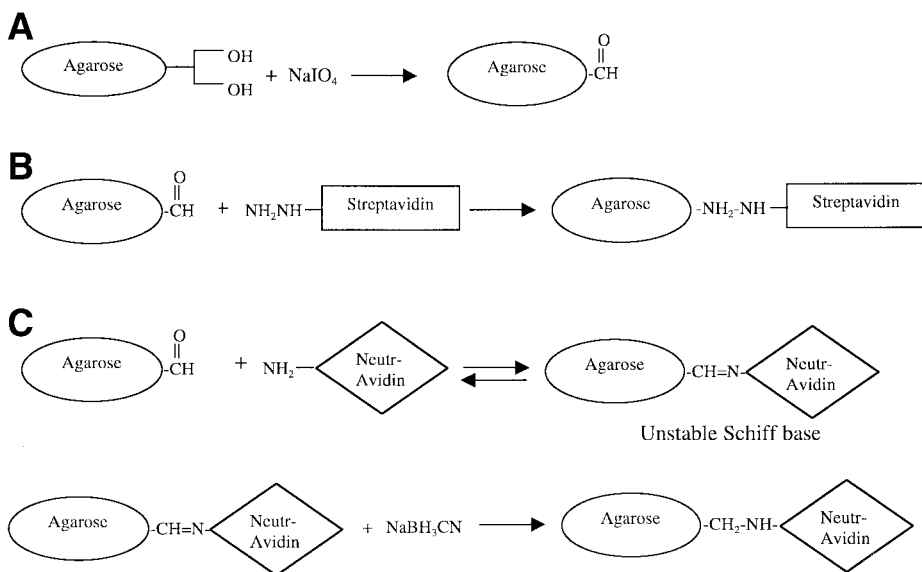
Fig. 1. Chemistry of agarose activation and conjugation to avidin derivatives. **(A)** Sodium meta-periodate oxidation of agarose to generate functional aldehydes. **(B)** Reaction of hydrazide groups of streptavidin–hydrazide with functional aldehydes of activated agarose. **(C)** Reaction of primary amine of NeutrAvidin with functional aldehydes of activated agarose to form unstable Schiff base, which is stabilized by reduction with NaBH$_3$CN in a reductive amination reaction.

8. Wash oxidized agarose for 10 min with 20 mL sterile dI water at 4°C
9. Repeat wash step 49 times, using a total of 1 L of water. Do not allow agarose to dry out.

3.2 Conjugation of Activated Agarose Substrate to Avidin

To uniformly incorporate biotinylated capture antibody into the oxidized matrix, agarose must be conjugated to avidin. We developed the assay using streptavidin, a biotin-binding analogue of egg-white avidin, gaining its prefix from its bacterium source, *Streptomyces avidinii*. The process of coupling agarose to streptavidin is detailed in **Subheading 3.2.1.** and in **Fig. 1B**. Streptavidin has been demonstrated to compete with extracellular matrix (ECM) proteins for binding to cell surface receptors of the integrin family through an RYD-containing sequence. This RYD-containing sequence mimics the RGD sequence common to several ECM proteins, such as fibronectin *(19–22)*. As potential uses for the EliCell system have evolved to include studies addressing eosinophil interactions with ECM proteins, we have begun to utilize alternatives

to streptavidin. One such substitute is NeutrAvidin® (Pierce), a carbohydrate-free modified avidin derivative. The process of coupling NeutrAvidin to agarose is described in **Subheading 3.2.2.** and in **Fig. 1C**.

3.2.1. Chemical Coupling of Streptavidin to Agarose

The hydrazide group of streptavidin–hydrazide is reacted with the oxidized agarose, to stably couple streptavidin to the activated agarose support matrix.

1. Cover agarose layer with 5 mL of 0.2 mg/mL streptavidin–hydrazide (diluted in water).
2. Incubate overnight at room temperature.
3. Repeat washing (**steps 8** and **9** in Subheading **3.1.**) to remove any unbound streptavidin.
4. OPTIONAL: the degree of streptavidin conjugation may be determined by analyzing an aliquot of the gel in benzoic acid at 500 nm using HABA reagent (refer to **Subheading 2.**).
5. Streptavidin–agarose may be re-solubilized at 70°C and aliquoted before storage. Aliquots may be stored up to 6 mo at 4°C.
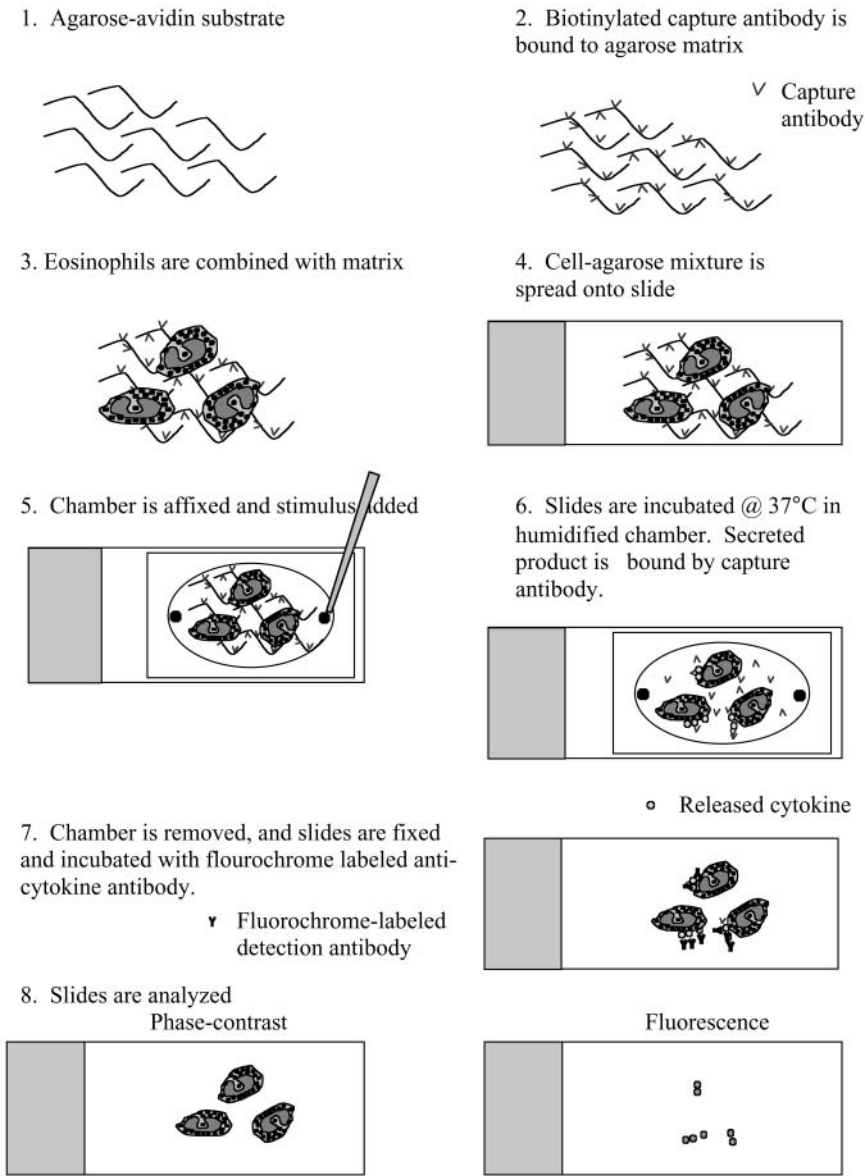
3.2.2. Chemical Coupling of NeutrAvidin to Agarose

Reactive aldehydes of the oxidized agarose are covalently coupled to available amine groups of neutravidin through a reductive amination reaction.

1. Cover agarose layer with sodium cyanoborohydride solution (32 mg $NaCNBH_3$ in 5 mL of 10 mM NaOH) plus 2 mg of NeutrAvidin (dissolved in 0.5 mL H_2O) and incubate overnight at 4°C. Because of the high toxicity of $NaCNBH_3$, this step should be performed in a fume hood.
2. To block unreacted groups, cover agarose with 5 mL of 1 M Tris and incubate for 2 h at room temperature.
3. Repeat washing **steps 8** and **9** from **Subheading 3.1.**
4. NeutrAvidin–agarose may be resolubilized at 70°C and aliquoted prior to storage.

3.3. EliCell Assay

Biotinylated cytokine-specific antibody is bound to avidin–agarose substrate in liquid state. Cells are exposed to stimulus, mixed with the liquid matrix and spread onto slides. As agarose solidifies, cells are immobilized in the capture antibody-containing substrate. The solid phase agarose–cell mixture is kept hydrated with stimulus containing medium and incubated for appropriate stimulation times. After stimulation, slides are incubated with fluorochrome-labeled cytokine-specific antibody to detect captured protein, and cell morphology and released product are analyzed in parallel by phase and fluorescence microscopy, respectively. The assay is illustrated in **Fig. 2**. *See* **Note 2** for a description of essential controls to be included in each experiment, and **Table 1** for sugges-

1. Agarose-avidin substrate

2. Biotinylated capture antibody is bound to agarose matrix

∨ Capture antibody

3. Eosinophils are combined with matrix

4. Cell-agarose mixture is spread onto slide

5. Chamber is affixed and stimulus added

6. Slides are incubated @ 37°C in humidified chamber. Secreted product is bound by capture antibody.

○ Released cytokine

7. Chamber is removed, and slides are fixed and incubated with flourochrome labeled anti-cytokine antibody.

 ⋎ Fluorochrome-labeled detection antibody

8. Slides are analyzed
     Phase-contrast

Fluorescence

Fig. 2. EliCell assay. Schematic of EliCell assay to detect release of a single cytokine.

tions on specific capture-detection antibody pairs and stimulus concentrations. For adaptation of protocol to detect two cytokines simultaneously, to detect released product in parallel with surface marker staining or for intracellular detection of non-released products refer to **Notes 3–5**, respectively.

Table 1
Examples of Capture–Detection Antibody Pairs and Stimulus Working Concentrations

Capture AB	Detection AB	Stimulus	Reference
Biotinylated goat polyclonal αhIL-4 (R&D Systems, cat. no. BAF204)	Mouse αhIL-4 (R&D Systems, Clone no. 3010.211)	Eotaxin (6 n*M*)	*15*
Biotinylated goat polyclonal αh RANTES (R&D Systems, cat. no. BAF278)	Mouse αhRANTES (R&D Systems, Clone no. 21418.211)	IFN-γ (500 U/mL)	*17*
Biotinylated goat polyclonal αh IL-12 (R&D Systems, cat. no. BAF219)	Mouse αhIL-12 (R&D Systems, Clone no. 24910.1)	αCD9 (2.5 μg/mL)	*18*

3.3.1. Binding of Capture Antibody to Substrate

1. Melt aliquot of streptavidin or NeutrAvidin-conjugated agarose at 70°C, then transfer to 37°C to maintain liquid state
2. Keeping at 37°C, combine the following in an Eppendorf tube (to determine final volume required multiply number of desired samples by 20 μL): 1 volume agarose, (+1/10 volume 10X concentrated RPMI-1640 medium), 1 volume biotinylated capture antibody (100 μg/mL in RPMI + 0.1% ovalbumin). Refer to **Note 6** for selection of appropriate capture-detection antibody pairs.
3. To above Eppendorf tube add 3 volumes of purified eosinophils (15×10^6 cells/mL in RPMI + 0.1% ovalbumin). Refer to **Note 7** for tips on eosinophil purification strategies.

3.3.2 Stimulation of Cells, Gelification of Substrate, and Incubation

1. For each stimulus, prepare in advance 1X and 10X working solutions. (Total volume required for 1X solution will be 400 μL × total number of slides, and for 10X solution will be 2 μL × total number of slides.)
2. Mix agarose/cell mixture (from **step 3** of **Subheading 3.3.1.**) thoroughly by aspirating gently with a plastic pipet tip.
3. In small Eppendorf carefully combine 20 μL agarose/cell mixture with 2 μL of 10X stimulus.
4. Using Ultra-microtips, gently spread (using surface tension) 22 μL stimulated agarose/cell mixture onto microscope slide. Avoid contacting slide surface with tip, as this may lead to cell damage.

5. Cover sample with perfusion chamber and place slide on tray atop hydrated pad. (Once chamber has been affixed, slides may remain at room temperature for several minutes while additional slides are prepared.)
6. Carefully pipet 400 µL of appropriate 1X stimulus over sample through a chamber access port, ensuring that chamber area is uniformly saturated.
7. Place tray in humidified incubator (37°C, 5% CO_2) for desired incubation time.
8. OPTIONAL: while slides are incubating, centrifuge fluorochrome-labeled detection antibody (15,000g for 30 min) to pellet any precipitate that may have formed during storage. Use supernatant to prepare working dilutions (**step 3**, **Subheading 3.3.3.**).

3.3.3. Fixation and Detection

1. After incubation, remove slides from incubator, carefully remove perfusion chambers and fix slides by immersion in 2% paraformaldehyde for 5 min at room temperature.
2. After fixation, wash slides for 10 min in HBSS –/– at room temperature with gentle agitation.
3. Prepare working dilutions of fluorochrome-labeled detection antibody in HBSS –/– (*see* **step 8, Subheading 3.3.2.**). Optimal concentrations must be determined for each detection antibody but generally range from 1 to 10 µg/mL. To determine total volume of detection antibody required, multiply number of slides by 400 µL. For suggestions on fluorochrome labeling of detection antibody, refer to **Note 1**.
4. Dry area immediately around agarose film on slides to prevent seepage of antibody, and place slides on hydrated pad. (Alternatively, pap pen may be used to outline staining area. However, care must be taken to avoid contacting agarose with pap pen.) DO NOT ALLOW SLIDES TO DRY.
5. Cover agarose film with 400 µL of diluted detection antibody and incubate 45 min at room temperature, protected from light.
6. After incubation with detection antibody, wash slides 3 × 10 min. in fresh HBSS –/– with gentle agitation.
7. After final wash, quickly dip slides in water to remove salt residue and allow slides to dry at room temperature, protected from light. (Slides may be left overnight to ensure drying.)
8. Once dry, slides should be mounted using aqueous mounting medium, coverslip affixed, and stored at room temperature in the dark until analysis. (Allowable storage time will depend upon fluorochrome used with detection antibody).

3.4. Analysis

The major advantage provided by the EliCell system is the ability to observe morphology and viability of individual cytokine-secreting cells. The next three subsections discuss analysis of results using fluorescence, phase-contrast and light microscopy.
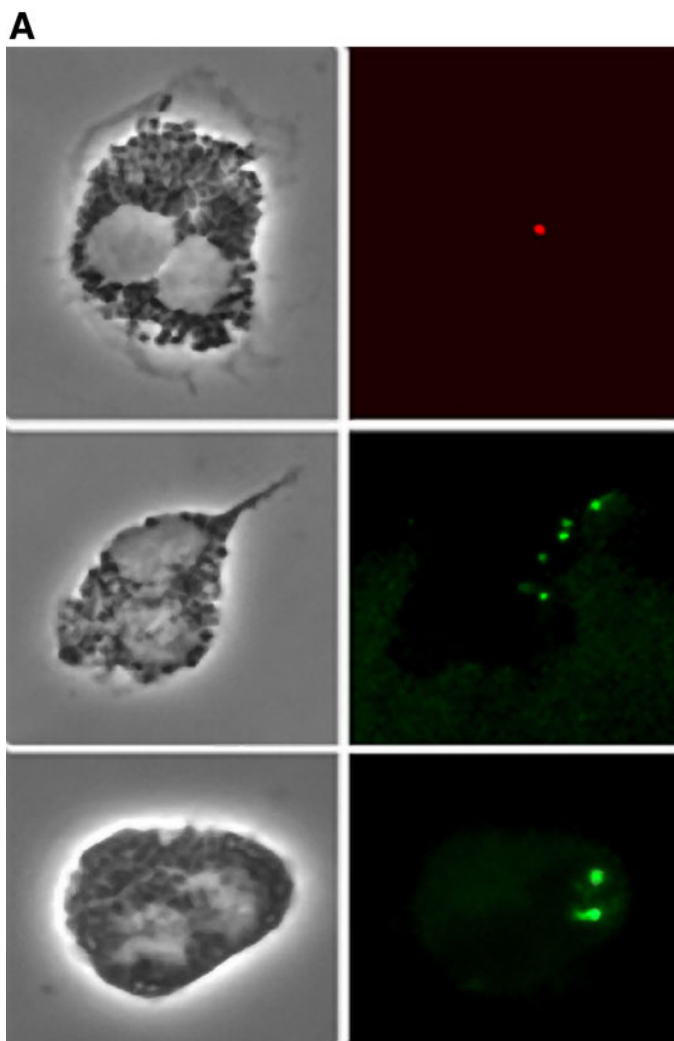
Fig. 3. Positive staining results using EliCell system. Detection of cytokine release from eosinophils stimulated with physiologic **(A)** or nonphysiologic **(B)** stimuli. In **(B)**, eosinophils were stimulated with 0.5 μM A23187. The bottom panel illustrates simultaneous detection of two cytokines labeled with Alexa 488 or Alexa 546. Digital pictures were taken using 100X magnification objective. *See* **Color Plate 7**, following page 50. *(Figure continues)*

3.4.1. Positive Staining Results

The detection of vesicle-released products using physiologic stimuli will appear as punctate spots at the cell surface (*see* Fig. 3A). A more diffuse staining pattern may indicate an insufficient concentration of capture antibody within the
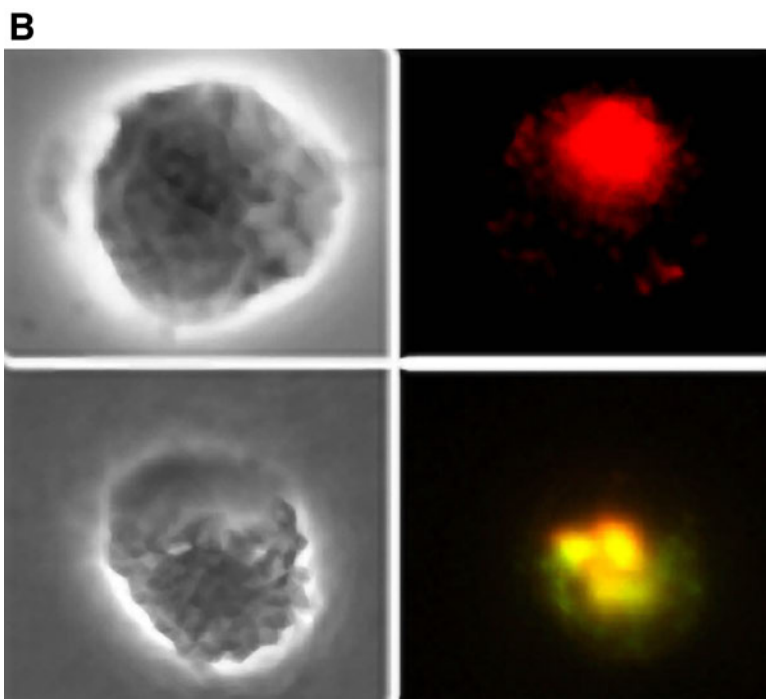
Fig. 3. *(continued)*

agarose matrix *(18)*. In contrast, detection of products released through cytolysis (i.e., following stimulation with a calcium ionophore) will appear as a much more robust staining, without discrete limits (**Fig. 3B**) and may be visualized in the absence of capture antibody, due to loss of membrane selectivity.

3.4.2. Staining Artifacts

It is essential that fluorescent product be analyzed in parallel with phase contrast analysis, to avoid counting staining artifacts as positive signal. In addition, appropriate controls must be included in each experiment (*see* **Note 2**). Common sources of staining artifacts arise from cellular autofluorescence exhibited by damaged or dying cells, non-specific antibody binding and permeabilization of cells. Generally, about 5% of the cells undergo degeneration and die by apoptosis during the EliCell assay. Examples of staining artifacts are shown in **Fig. 4**.

3.4.3. Cell Viability and Morphological Analysis

A major benefit of the EliCell system is the maintenance of cell viability (greater than 90%) even after fixation and detection steps. Viability of cells may
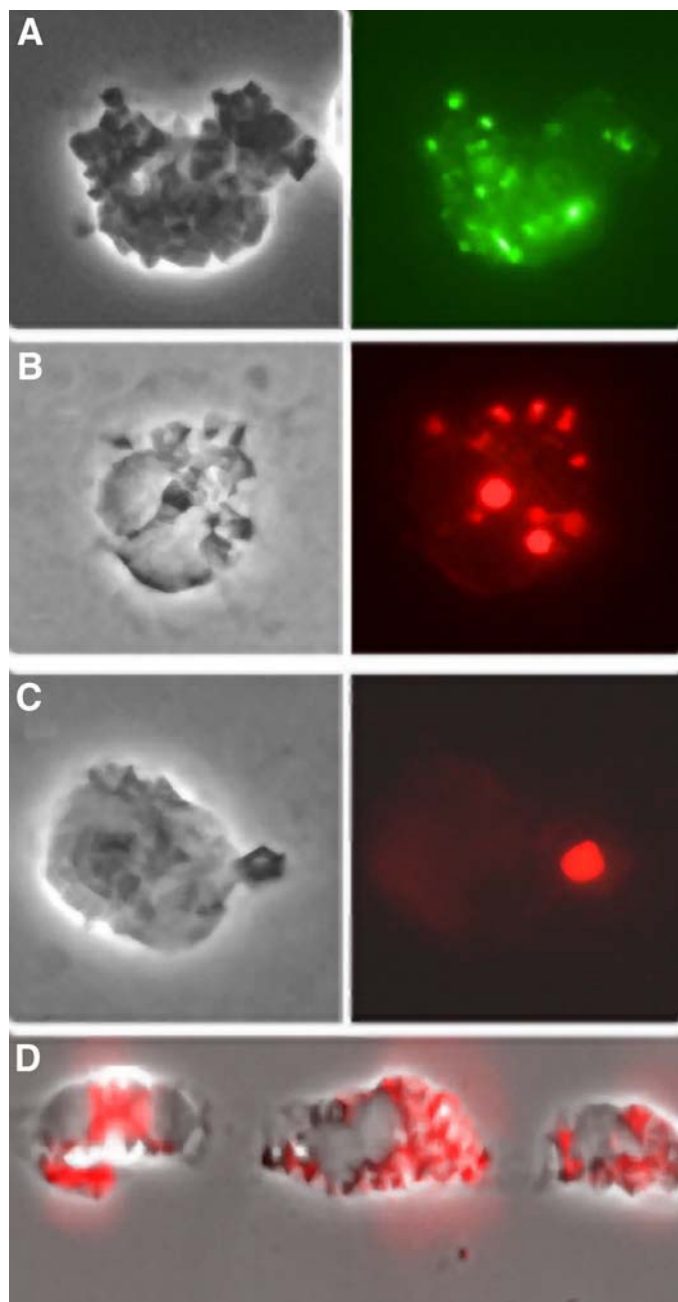
Fig. 4. Staining artifacts using EliCell system. Phase-contrast and fluorescence microscopy of identical fields of eosinophils incubated in EliCell preparations. Damaged (**A, B,** and **C**) or permeabilized (**D**) cells show nonspecific staining. In **D,** the image was overlaid. Digital pictures were taken using 100X magnification objective. *See* **Color Plate 8**, following page 50.
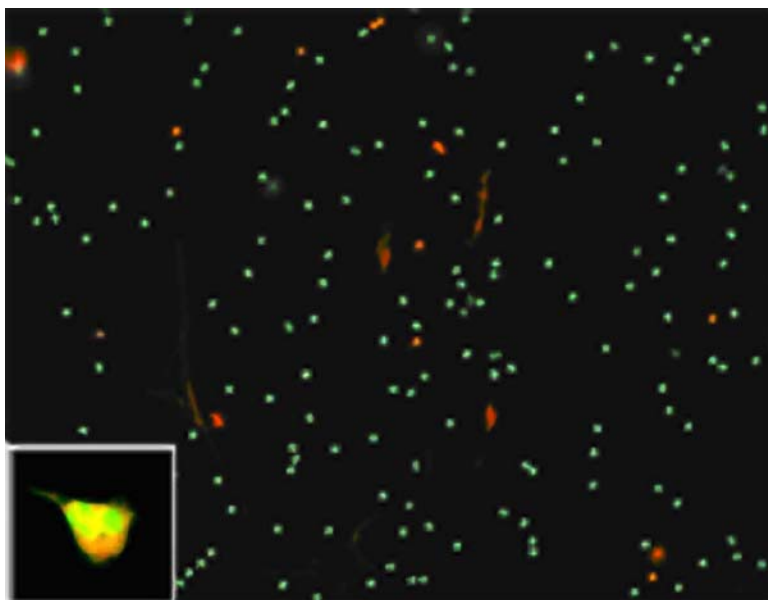
Fig. 5. Viability of cells after EliCell assay. EliCell preparation of eosinophils stained with acridine orange/ethidium bromide mixture after fixation. Most cells show green fluorescent nucleus indicative of cell viability. Digital pictures were taken using 100X magnification objective. *See* **Color Plate 9**, following page 50.

be monitored in the EliCell system using ethidium bromide staining as outlined in **Subheading 3.4.3.1.** Another key feature of the EliCell system is the ability to analyze cellular morphology throughout experimental manipulations. In addition to analysis by phase-contrast (described in **Figs. 3** and **4**), cell morphology may also be studied by bright-field microscopy. Preparation of cells for this purpose is described in **Subheading 3.4.3.2**.

3.4.3.1. MONITORING CELL VIABILITY

1. After a 5-min fixation of slides in 2% PFO (**step 1, Subheading 3.3.3.**), wash once in HBSS –/–.
2. Add 300 µL of 1:1000 dilution of acridine orange:ethidium bromide mixture (*see* **Subheading 2.**).
3. Coverslip and analyze fluorescence using FITC and rhodamine filters without allowing cells to dry. Live cells will appear slightly red with green nuclear staining, while nuclei of dead cells will intercalate ethidium bromide and appear red (**Fig. 5**).
4. Alternatively, cell viability may also be analyzed adding the acridine orange:ethidium bromide solution directly into the chamber access port after incubation with stimulus. In this case, an inverted microscope will be necessary for slide analysis.
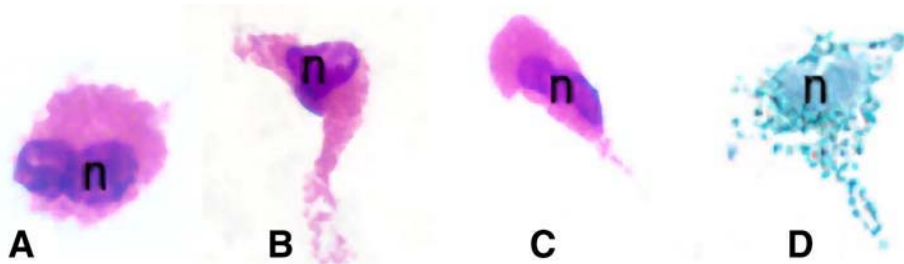
Fig. 6. Morphology of eosinophils during EliCell assay. Light micrographs of eosinophils observed in the EliCell system before **(A)** and after stimulation with eotaxin **(B–D)**. Morphological changes characterized by cell elongation are clearly seen in stimulated cells. Cells were stained with Hema 3 **(A–C)** or fast green/hematoxylin **(D)**. n, nucleus. Digital pictures were taken using 100X magnification objective. *See* **Color Plate 10**, following page 50.

3.4.3.2. MORPHOLOGICAL ANALYSIS

After the desired EliCell step to be analyzed, cells may be prepared as follows:

1. Fix the agarose film containing cells in methanol for 1 min. Fixation is accomplished by either slide immersion in the fixative (without chamber) or by adding the fixative directly into the chamber access port.
2. Stain the cells with acid/basic dyes such as fast green/hematoxylin (0.2% fast green in 70% ethanol for 20 min followed by hematoxylin for 5 s) or Hema 3® kit (Fisher Scientific).
3. Mount and analyze the slides using a bright-field microscope with 100X objective.
4. Alternatively, cells may be fixed with 2% paraformaldehyde for 5 min and stained with 2% chromotrope 2R for 5 min.

Eosinophils in the EliCell system may show different morphologies, ranging from round to highly polarized cells. Generally, after stimulation, a great proportion of eosinophils are seen as elongated cells (**Fig. 6**). This morphological change indicates that the cells are activated. Refer to **Note 8** for adaptation of protocol for additional cytochemical analyses.

4. Notes

1. When choosing a fluorochrome for detection antibody labeling, it is important to consider stability of the signal, as well as its staining intensity. We routinely use Alexa Fluor 546 (red) or Alexa Fluor 488 (green). Protein labeling kits are available from Molecular Probes (cat. no. A-10237 and A-10235 for Alexa 546 and Alexa 488, respectively). Allow approx 2 h for the labeling procedure.

2. Because of the staining artifacts discussed in **Subheading 3.4.2.** and to control for quality of purified eosinophils, the following controls must be included in each experiment, for each condition: (1) medium alone (no stimulus); (2) isotype control fluorochrome labeled detection antibody; (3) capture antibody omitted from agarose or replacement with irrelevant capture antibody.

3. The EliCell assay may be adapted to detect two cytokines simultaneously. In this case two capture antibodies will be combined with the avidin-agarose in **step 2** of **Subheading 3.3.1**. To maintain appropriate matrix consistency, the total volume added must be equivalent to 1 volume. Therefore, add 0.5 volume each of 2X stock biotinylated capture antibody. Fluorescently labeled detection antibodies should be chosen which do not cross-react with each other or either capture antibody, and whose emission spectra do not overlap. Diluted detection antibodies may be added simultaneously (**step 5, Subheading 3.3.3.**) in a total volume of 400 μL. (*See* **Fig. 3B** for illustration of positive signal.)

4. In addition, the EliCell assay may be adapted for the simultaneous detection of surface marker expression and released product. It is important to first determine whether fixation alters the antigenicity of the surface marker in question. If fixation does not alter ability of detection antibody to bind surface marker, then EliCell procedure may be followed as described, with the addition of a fluorochrome labeled antibody against surface marker added simultaneously with anti-cytokine detection antibody. Alternatively, slides may be incubated with fluorochrome-labeled antibody against the surface marker prior to fixation in 2% PFO (**step 1, Subheading 3.3.3.**). Slides should then be washed in HBSS –/– before continuing with fixation and subsequent staining with anti-cytokine detection antibody.

5. The EliCell system may be modified to immunolocalize intracellular products formed by eosinophils (i.e., the principal cysteinyl leukotriene LTC_4). In this case, the agarose is prepared without avidin and the cells must be permeabilized before detection with a specific fluorochrome-labeled antibody. For LTC_4 detection, cells are prepared as for conventional EliCell assay, with the following modifications *(23)*: (1) Eosinophils are embedded in the agarose matrix without binding of capture antibody to substrate. 1 volume of RPMI + 0.1% OVA should be added to tube to compensate for depleted volume (**step 2, Subheading 3.3.1.**). (2) After stimulation, cells are permeabilized and fixed with carbodiimide chemistry that induces cross-links between carboxyl group of newly synthesized LTC_4 to amines of adjacent proteins. (3) Fluorescently labeled detection antibody (i.e., Alexa488-labeled Ab specific for LTC_4) is used to detect and localize the immobilized leukotriene.

6. When choosing capture and detection antibody pairs, it is important to choose antibodies which react with unique epitopes of the target cytokine. We generally choose a polyclonal capture antibody and a monoclonal detection antibody to decrease the probability of antibody hindrance.

7. We routinely isolate eosinophils from human peripheral blood by Hypaque-Ficoll separation followed by negative selection using an immunomagnetic depletion protocol (Stem Cell Technologies, Vancouver, BC; cat. no. 14156). Much of the success of the EliCell assay will depend upon the initial quality of cells. Therefore, it
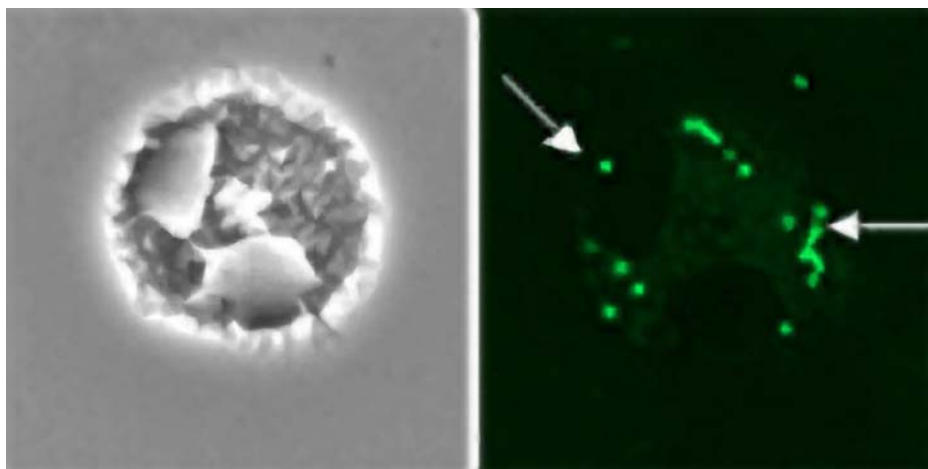
Fig. 7. Intracellular lipid body staining using EliCell system. Phase-contrast and fluorescence microscopy of identical field of chemokine-stimulated eosinophil in an EliCell preparation. Cytoplasmic lipid bodies are indicated (arrows). Cells were stained with BODIPY. Digital pictures were taken using 100X magnification. *See* **Color Plate 11**, following page 50.

is essential that great care be taken during the eosinophil purification process. Specifically, hypotonic saline solution should be used for red blood cell lysis (avoid using NH$_4$Cl solution). HBSS without calcium or magnesium should be used throughout purification process to avoid activation of eosinophils. In addition, it has been our experience that hypodense eosinophils are more fragile than normodense cells. Therefore if possible, care should be taken to recover only the normodense layer after Ficoll separation.

8. In addition to immunolocalization analyses, the EliCell assay may be useful for multiple cytochemical studies. For instance, viable eosinophils embedded in the agar matrix (prepared without avidin incubation) may be stimulated to induce lipid body formation, which may be detected by Nile Red or BODIPY® (Molecular Probes) staining (**Fig. 7**).

Acknowlegments

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