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## Rapid densitometric determination of cell migration and cell adhesion in a microchemotaxis chamber

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A new rapid staining and measuring method has been developed for the quantification of migrated cells in a microchemotaxis chamber. The migrated cells were, after staining, evaluated by a transmission densitometer. The method introduced here is more accurate and faster than those described previously. In addition the technique can be used to determine the adherent capacity of cells.

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*Key words:* Chemotaxis assay; Adherence assay; Densitometer; Quantification

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### Introduction

The measurement of the chemotactic responses of leukocytes and tumor cells provides a very important evaluation of cell function. A variety of methods has been developed to study these responses (Wilkinson, 1986; Bignold, 1988), and it has been demonstrated that a microchemotaxis chamber, equipped with polyvinylpyrrolidone-free membranes, is a useful method for quantifying *in vitro* migration, especially if only small numbers of cells are available (Falk et al., 1980; Harvath et al., 1980). However, a major drawback of such microchemotaxis chamber techniques is that they tend to be time-consuming.

A number of techniques are now available for the measurement of migratory responses, includ-

ing counting the cells under a microscope, or automatically using an Optomax image analyzer (Minkin et al., 1985), or using ELISA and radio-labeling techniques (Gallin et al., 1973; Sorg et al., 1982; Sims et al., 1985).

In this report we describe a new accurate method for measuring the number of migrated cells which is much less time-consuming.

### Materials and methods

#### *Cells*

Human peripheral blood monocytes and granulocytes were isolated as described previously (Figdor et al., 1982, 1984) from the blood of healthy blood donors. First, the cells from each 500 ml donation were separated by density centrifugation with a blood component separator. Subsequently, the cells were fractionated into lymphocytes, monocytes and granulocytes by centrifugal elutriation. The monocyte fraction was over 95% pure as judged by non-specific esterase staining and contained more than 98% viable cells. The granulocyte fraction was over 98% pure as

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*Abbreviations:* fMLP, *N*-formyl-L-methionyl-L-leucine-L-phenylalanine; LFA-1, leukocyte function associated antigen-1; PVP, polyvinylpyrrolidone.

judged by May-Grünwald-Giemsa staining. The OM-30 melanoma cell line is a long-term culture of melanoma cells (skin metastasis) initially selected for its capacity to adhere to plastic surfaces within 30 min.

#### *Chemotaxis assay*

Directed monocyte, granulocyte and melanoma migration were measured in a 48-well microchemotaxis chamber (Falk et al., 1980) (Neuroprobe, Bethesda, MD). The chamber consisted of an upper and lower compartment divided into two parts by a 10  $\mu\text{m}$  thick polyvinylpyrrolidone-free polycarbonate membrane filter (Nucleopore, Pleasanton, CA) with 5  $\mu\text{m}$  pores for monocytes and granulocytes and with 8  $\mu\text{m}$  pores for OM-30 cells. The lower compartment was filled with 25  $\mu\text{l}$  of medium with or without fMLP (Sigma Chemical Co., St. Louis, MO) as chemoattractant for monocytes and granulocytes or with laminin (Bethesda Research Laboratories, Gaithersburg, MD) as chemoattractant for the OM-30 melanoma cells. The chambers were incubated for 120 min at 37°C, 5% CO<sub>2</sub> on a waterlevel construction. After incubation the non-migratory cells were removed from the upper side of the filter and the number of migrated cells that adhered to the bottom side of the filter was determined as described below.

#### *Adhesion assay*

Monocytes or melanoma cells were allowed to adhere to a PVP-free polycarbonate membrane filter (5  $\mu\text{m}$  pores) for 30 and 60 min, respectively, in the chemotaxis chamber with empty lower compartment. After incubation the filter was removed and washed thoroughly by agitation in a petri dish filled with PBS. Subsequently the cells were stained after fixation (see below). In some experiments the filter was coated with laminin (100  $\mu\text{g}/\text{ml}$ ) or fibronectin (150  $\mu\text{g}/\text{ml}$ ) prior to use. Inhibition of adhesion was investigated by the addition of a monoclonal antibody (mAb) CLB-LFA-1/1, which is directed against the common  $\beta$  chain of the adhesion molecules LFA-1, CR3 and p150,95 (Miedema et al., 1984). The mAb Gepi-1 (Keizer et al., 1986) was used as a control antibody.

#### *Calibration curves*

Calibration curves were prepared by adding known numbers of cells to the upper compartment

of a microchemotaxis chamber without filling the lower compartment. The cells were allowed to adhere to the filter. Subsequently the filter was dried on tissue paper, fixed and stained as described below.

#### *Staining procedures*

After removal of non-migrated cells the filter was fixed in methanol for 10 s and allowed to dry for 1 min. Subsequently the cells were stained in a two-step staining procedure. First the filter was incubated for 4 min in Coomassie brilliant blue solution (5 g, Coomassie brilliant (blue R250) in 908 ml methanol + 908 ml H<sub>2</sub>O + 184 ml acetic acid) and destained for 30 s (acetic acid:ethanol:H<sub>2</sub>O (7.5:20:72.5)). Secondly the filter was incubated in a 1% crystal violet solution in H<sub>2</sub>O for 4 min, washed three times in distilled water and allowed to dry.

#### *Quantification of migration*

After staining the migrated cells which adhered to the lower part of the filter were evaluated by means of a transmission densitometer (Macbeth TD 904, Newburgh, NY). This densitometer was a special purpose instrument designed for prepress operation in the printing industry. The optical density was defined according to the following equation:

$$D = 10 \log \frac{I_0}{I_t}$$

In which  $D$  was the optical density,  $I_0$  was the total incident light (100%) and  $I_t$  was the transmitted light. The optical density of the stained cells was measured and interpolated on the calibration curve to calculate the migration percentages. We adjusted the standard 2 mm aperture of the densitometer to a 2.7 mm aperture, which equalled a surface area of 80% of the well. The transmitted light was filtered by an optical filter (Kodak wratten gelatin, filter no. 90, Rochester, NY) (see Fig. 1).

In some experiments we compared the densitometric measurements with microscopic counting. In these cases at least ten randomly chosen high power magnification fields (magnification field

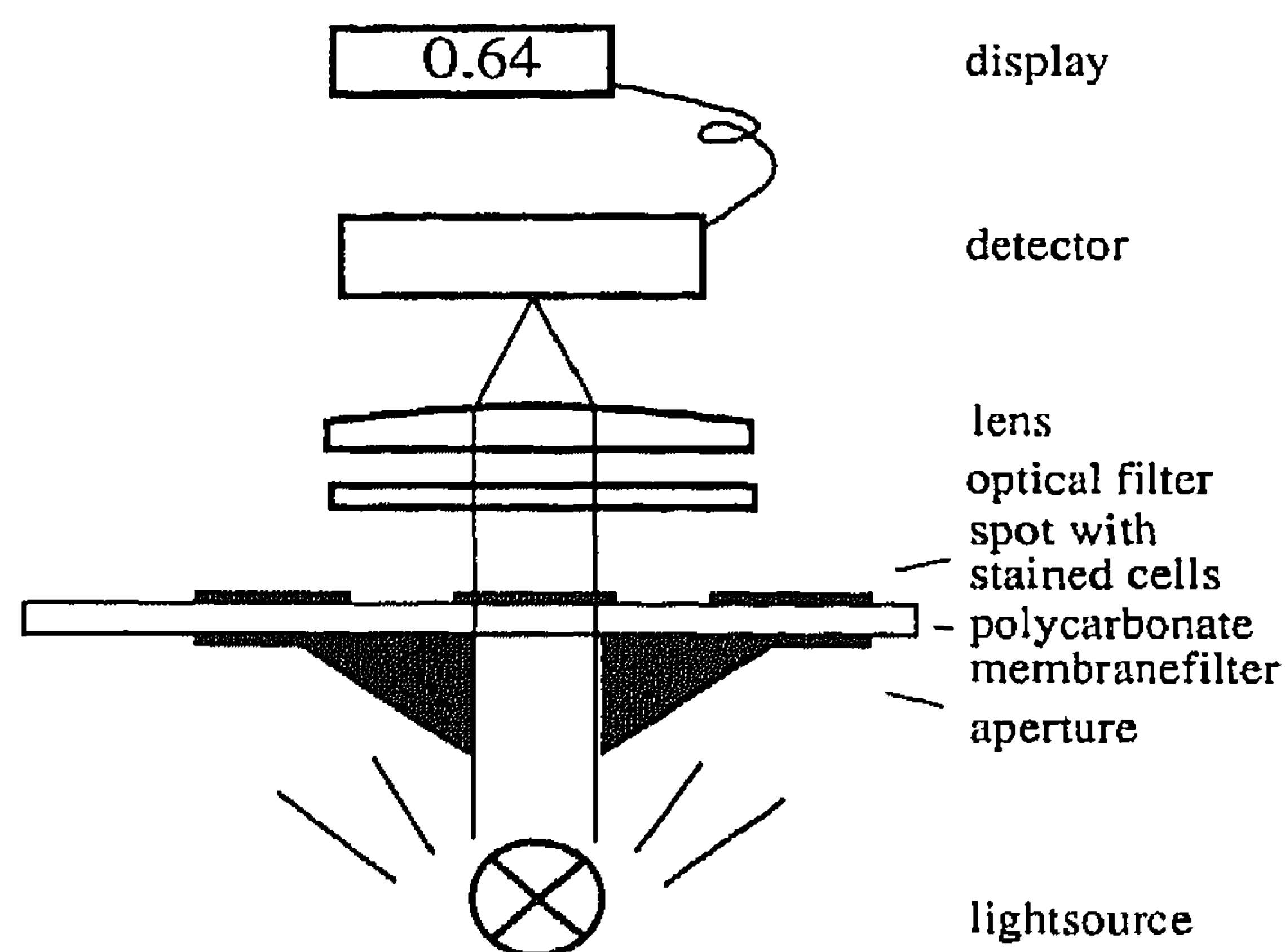


Fig. 1. Diagram of the densitometer.

400 $\times$ ) were counted and the percentage of migrated cells calculated.

## Results

### Staining

Staining of cells was required to permit densitometric determination of the number of cells that

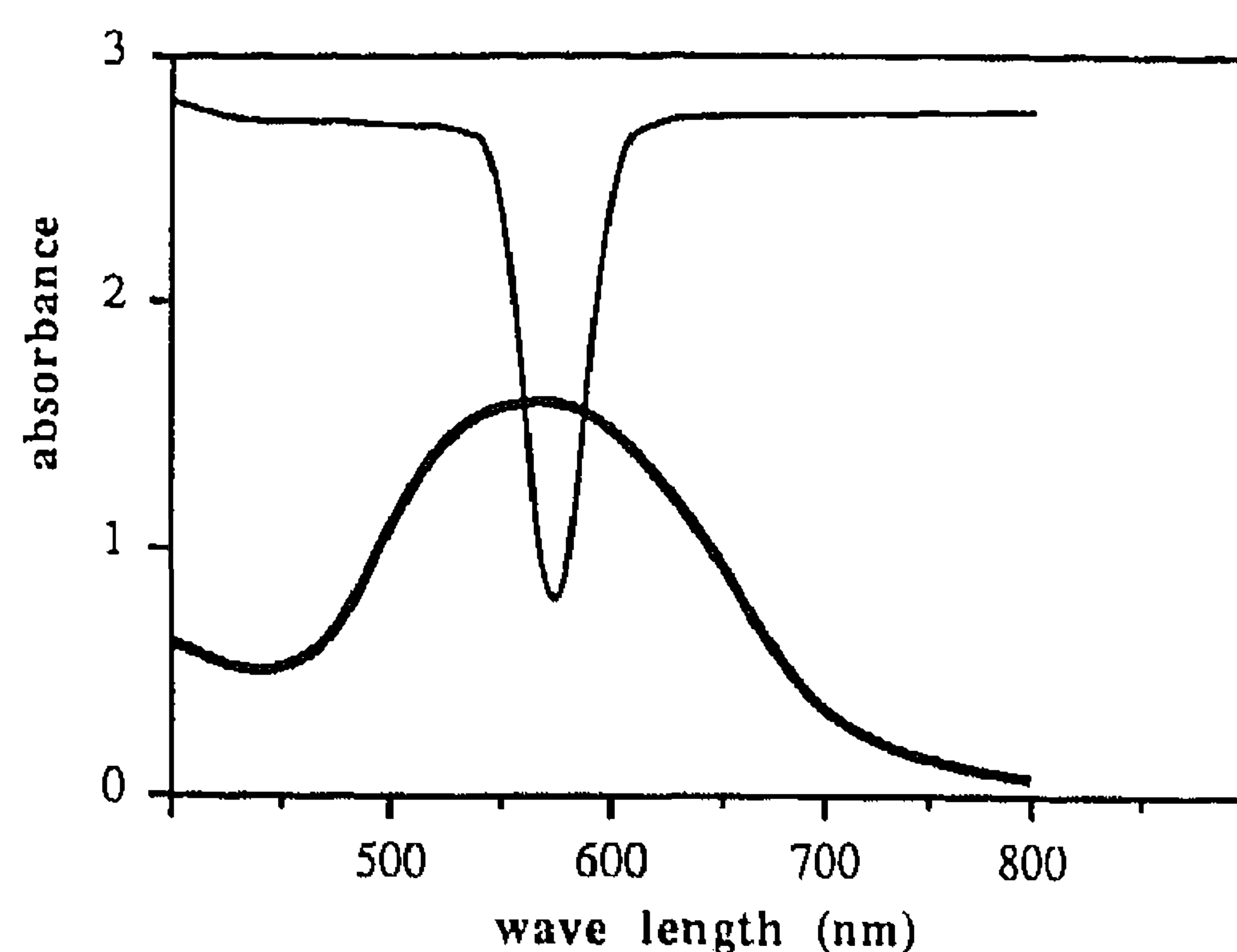


Fig. 2. Light absorbance spectra of stained cells and the optical filter. Monocytes ( $50 \times 10^3$ /well) were allowed to adhere to a PVP-free polycarbonate filter for 30 min at 37°C, 5% CO<sub>2</sub>, 100% humidity. After staining the cells were measured in a spectrophotometer. The thick line represents the spectrum of the adherent cells and the thin line that of the optical filter (no. 90, Kodak wratten gelatin).

were adherent to the membrane filter. It appeared that the existing staining methods were not suitable for accurate counting of low cell numbers by densitometry. To improve the absorbance of inci-

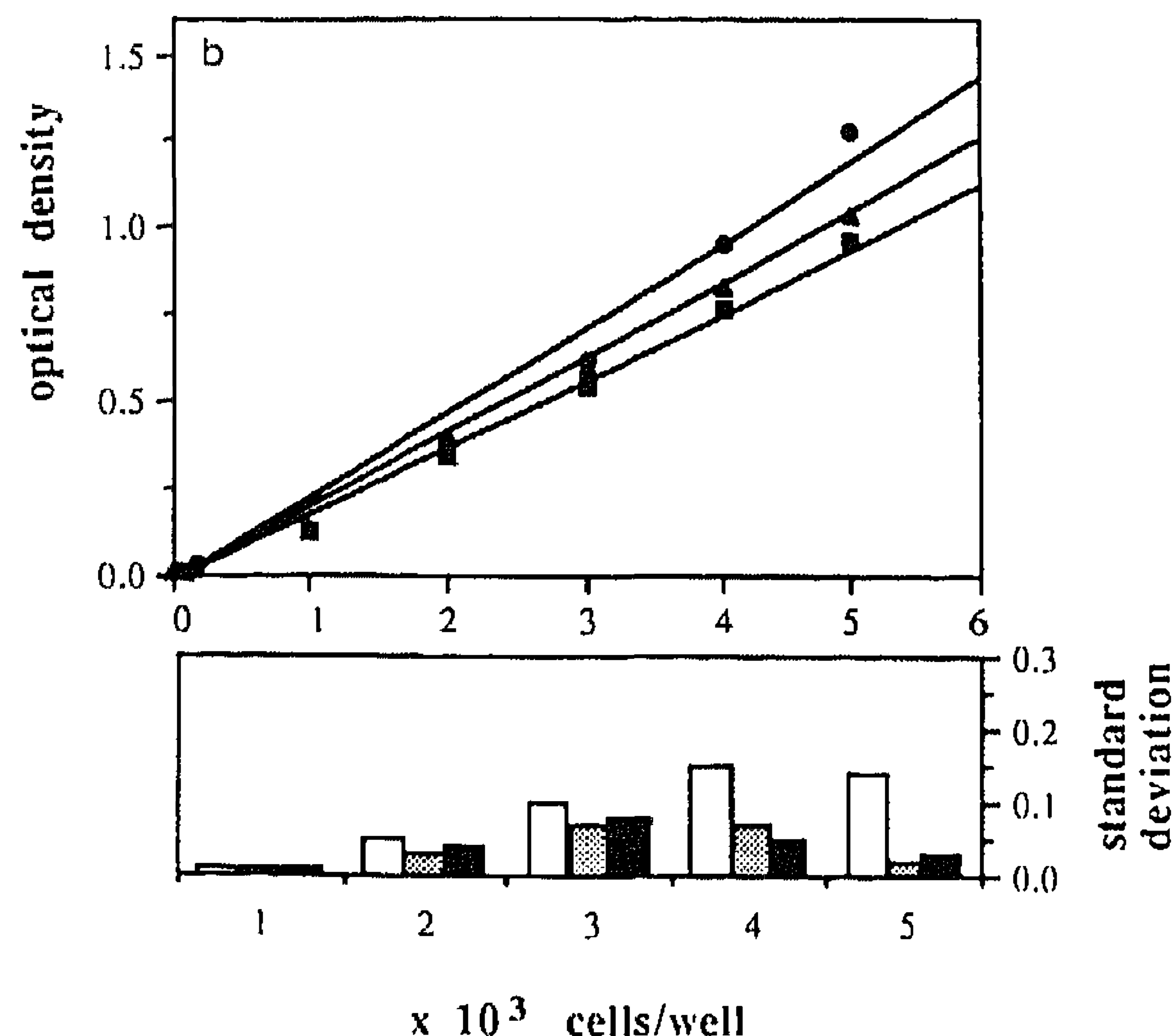
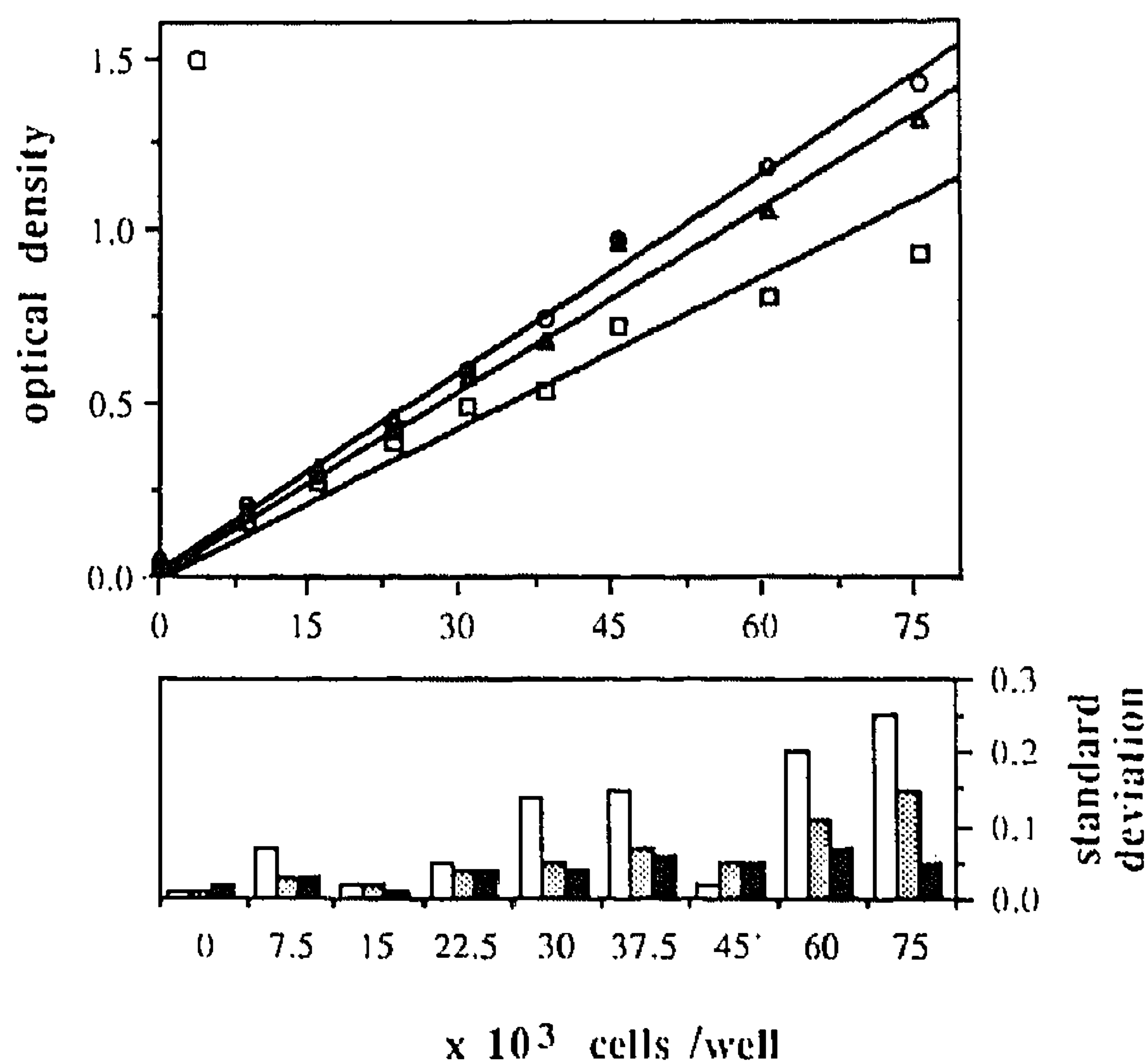


Fig. 3. *a*: Calibration curves of monocytes, measured with an aperture of: 1 mm ( $\circ$ ), and corresponding standard deviation (white bars); 2.7 mm ( $\Delta$ ), and corresponding standard deviation (grey bars); 3 mm ( $\square$ ), and corresponding standard deviation (black bars). Increasing numbers of monocytes were allowed to adhere for 45 min and measured with a densitometer as described in the materials and methods section. A representative experiment is shown. *b*: Calibration curves of melanoma cells (OM-30) measured with an aperture of: 1 mm ( $\bullet$ ), with corresponding standard deviation (white bars); 2.7 ( $\blacktriangle$ ), with corresponding standard deviation (grey bars) 3 mm ( $\blacksquare$ ), with corresponding standard deviation (black bars). Increasing numbers of OM-30 melanoma cells were allowed to adhere for 60 min and measured with a densitometer as described. A representative experiment is shown. Similar results were obtained in eight independent experiments with a mean slope of  $4.4 \pm 0.6$  ( $10^3$  cells/density) measured with an aperture of 2.7 mm.

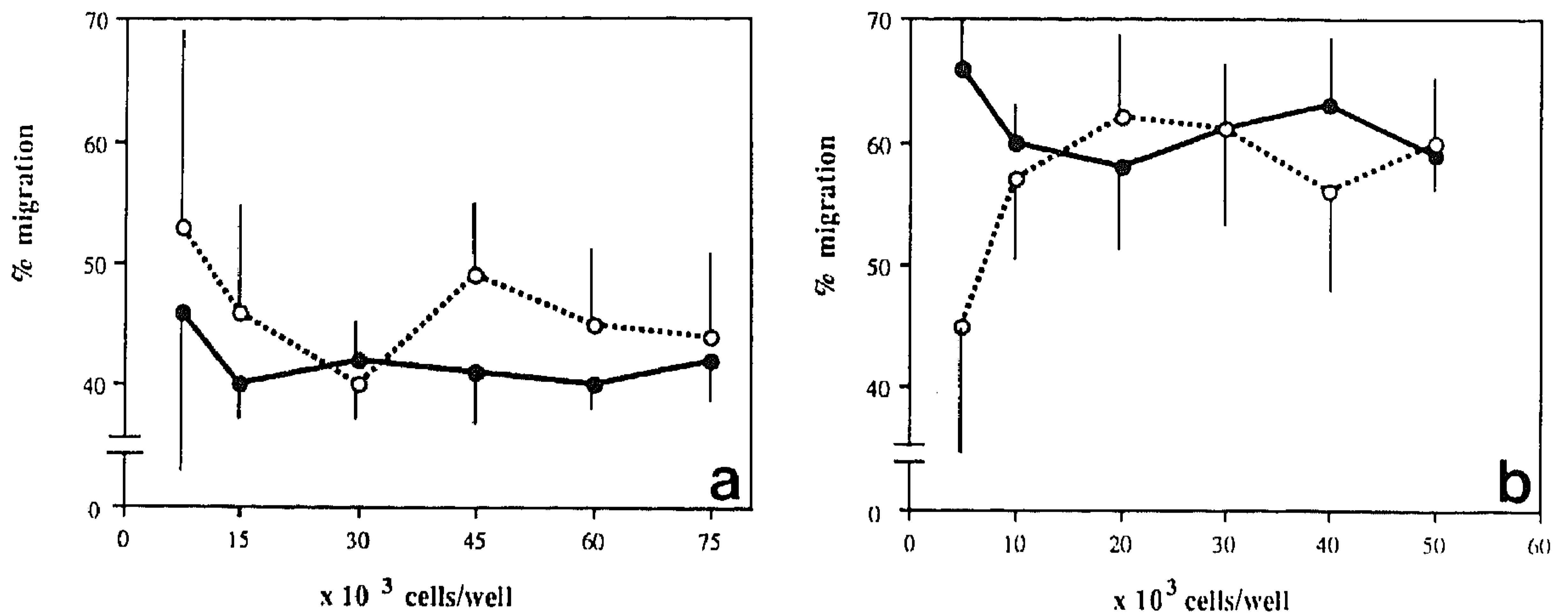


Fig. 4. Migration of monocytes and granulocytes. Increasing numbers of monocytes (a) and granulocytes (b) were tested for their migration capacity. The migrated cells were measured with densitometry (●—●) and microscopy (○-----○).  $10^{-8}$  M and  $10^{-7}$  M fMLP were used as the chemoattractants for monocytes and granulocytes, respectively.

dent light by the cells, we developed a staining procedure which allowed densitometric measurement of less than 15 000 cells. Optimal results were obtained when the membrane filters containing the migrated cells were stained in two steps by Coomassie brilliant blue followed by crystal violet. To assess the optimal light absorbance of stained cells, they were measured with a spectrophotometer as shown in Fig. 2. These measurements resulted in the choice of an optical filter of 570 nm (Kodak wratten gelatin, no. 90) to optimize the density measurements.

#### Calibration

In order to ascertain the number of migrated or adherent cells in a microchemotaxis chamber by means of densitometry, calibration curves were prepared. The results in Figs. 3a and 3b demonstrate that the optical density was directly proportional to the cell number. To further optimize the sensitivity of the measurement we investigated the aperture of the sensor, since the possibility existed that the cells were non randomly distributed, especially in the periphery where the filter was in contact with the polycarbonate of the chemotaxis chamber. Although the steepness of the calibration curves of monocytes and melanoma cells do not differ significantly at various apertures (1, 2.7, 3 mm) the lowest standard deviation and the highest coefficient of correlation was obtained with

an aperture of 2.7 mm. The coefficient of correlation was 0.988 and 0.999 for monocytes and melanoma cells, respectively. Similar results were obtained for granulocytes (results not shown). Therefore all other experiments were carried out with an aperture of 2.7 mm.

#### Migration

The minimal number of cells required for reliable quantification was determined in the experiment shown in Fig. 4. In the upper well of the chemotaxis chamber decreasing amounts of cells were exposed to the optimal concentrations of the chemoattractant fMLP or laminin. From these results it was concluded that the minimal number of cells which could be used was 15 000 and 10 000 for monocytes and granulocytes, respectively, and only 4000 for OM-30 melanoma cells (results not shown). In addition we compared the values obtained by densitometry with those obtained by microscopic examination. The results in Fig. 4 clearly show that the data obtained microscopically are less accurate especially if small numbers of cells are used. From these results we concluded that the densitometric determination was more accurate.

#### Adhesion

Finally, we investigated whether the microchemotaxis chamber was also able to measure the

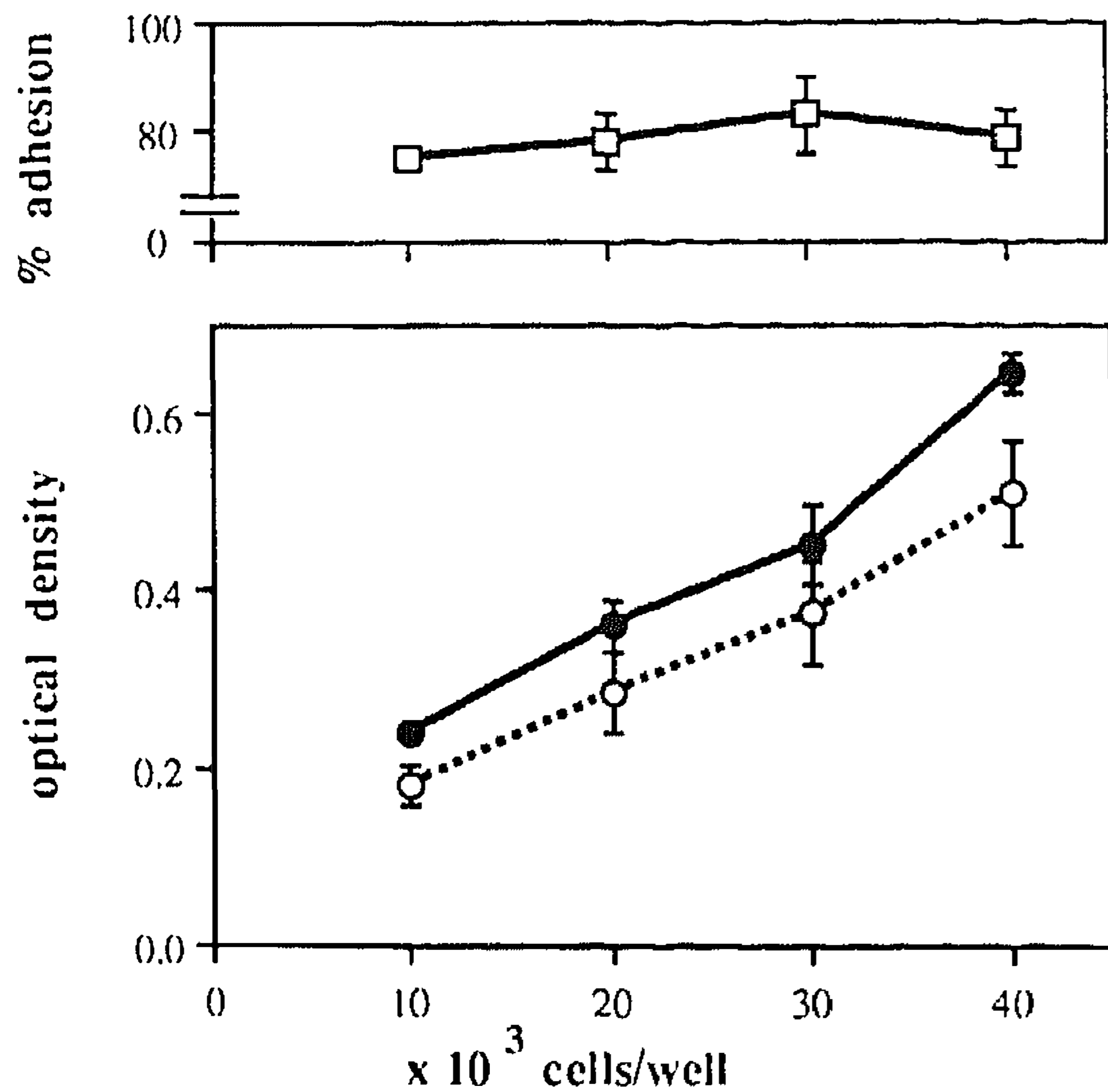


Fig. 5. Adhesion of monocytes. Increasing numbers of monocytes were allowed to adhere for 60 min. The adherent cells were measured with densitometry: (●—●), total number of the cells added to the well; (○-----○), adherent cells. The calculated adhesion percentages are shown on top of this figure.

adherent capacity of monocytes and melanoma cells. Fig. 5 shows that the optical density was directly proportional to the number of cells. Washing the filter as described in the Materials and methods section resulted in the same percentage of adhesion, regardless of the number of cells added, up to a maximum of 75 000 monocytes/well (well area completely covered). Fur-

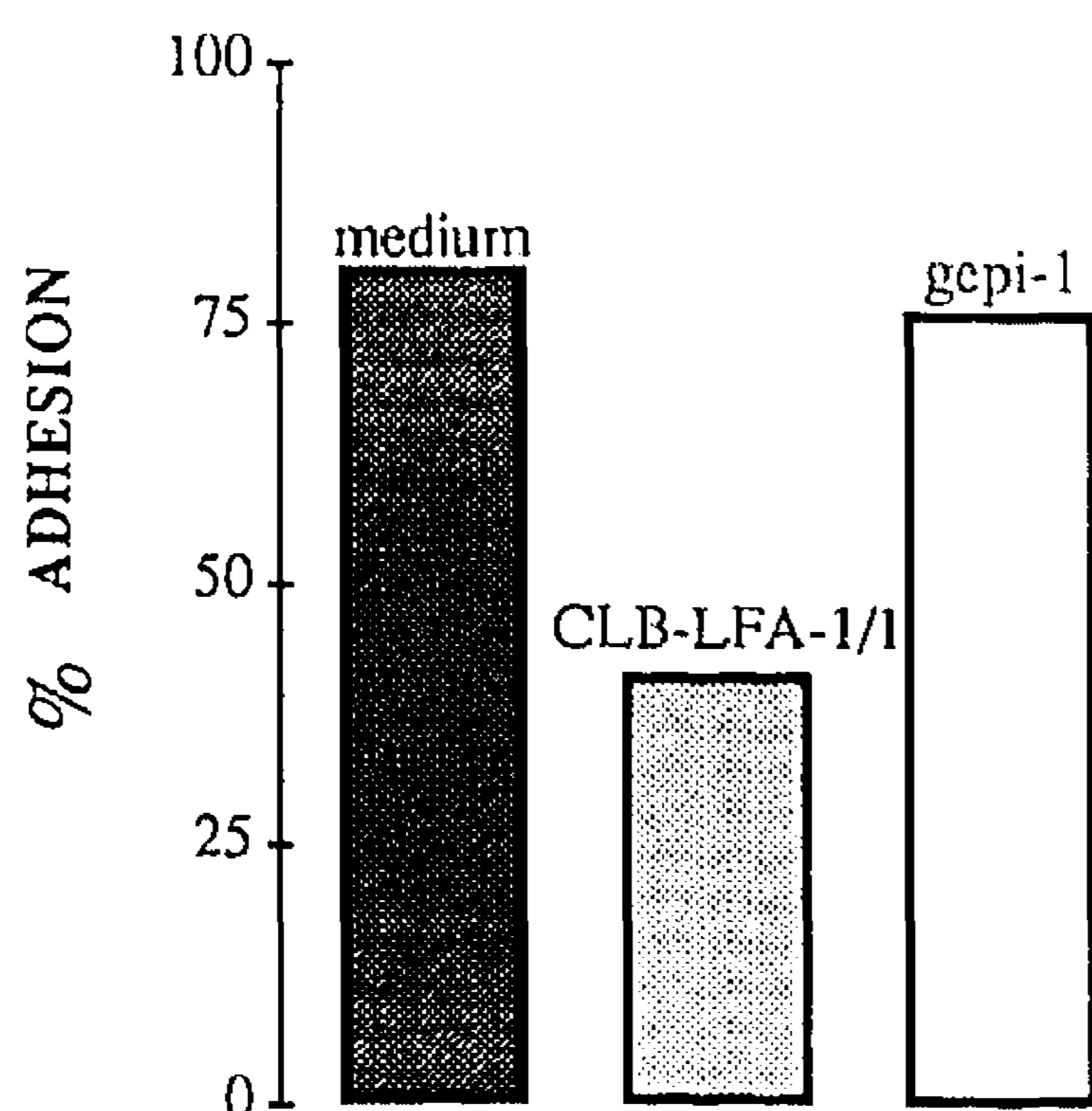


Fig. 6. Inhibition of adhesion. The adherence capacity of monocytes was measured in an adhesion assay as described in the materials and methods section in the presence of mAbs (CLB-LFA-1/1, Gepi-1), dilution 1/250 ascites.  $4 \times 10^4$  monocytes were added to each individual well.

thermore, as shown in Fig. 6, inhibition of adhesion could be measured by the addition of antibodies directed against the adhesion molecule LFA-1, but not by Gepi-1 which recognizes an antigen that is not involved in adhesion. From these data we concluded that the microchemotaxis chamber and densitometer were suitable for determining the adherent capacity of cells. Moreover, the adherence assay could also be used with melanoma cells on different substrates (results not shown).

## Discussion

The combined use of a new staining procedure and densitometry provides a simple method to quantify the migration and adhesion of cells. The assay including staining, takes only 20–30 min for 48 samples and a high degree of accuracy can be achieved even with low cell numbers ( $\leq 15\,000$  cells/well).

Various other microchemotaxis chamber methods have been developed to determine the number of migrating cells. The use of radiolabeled cells requires special precautions and is time-consuming (washing after labeling). Moreover, because of this preincubation step it is not possible to test fresh cells. ELISA techniques are laborious since they are subject to many manipulations. Furthermore, antibodies directed against cell surface antigens are generally employed to determine the number of migrating cells and antigen expression can differ in a cell suspension, thereby influencing the results. In addition different antibodies may be required to measure responses by distinct cell types. Evaluation by light microscopy of the number of migrating cells is extremely time-consuming, whether by manual or automatic (image analyzer) counting. Furthermore, these techniques measure only a small part of the surface areas of the wells, 12.5% in the case of image analyzer measurements and less than 2% with manual examination (Falk et al., 1980; Minkin et al., 1985). Our results demonstrated that the densitometric approach guarantees high accuracy since approximately 80% of the surface area of the well is included in the measurement. We observed (results not shown) that the cell density on the filter

was not always homogeneous during the migration/adhesion assay. By measuring 80% of the total well area, this potential problem is circumvented.

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