

Quantifying Lamella Dynamics of Cultured Cells by SACED, a New Computer-Assisted Motion Analysis

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Formation of lamellipodia and the retraction of ruffles are essential activities during motility and migration of eukaryotic cells. We have developed a computer-assisted stroboscopic method for the continuous observation of cell dynamics (stroboscopic analysis of cell dynamics, SACED) that allows one to analyze changes in lamellipodia protrusion and ruffle retraction with high resolution in space and time. To demonstrate the potential of this method we analyzed keratinocytes in culture, unstimulated or stimulated with epidermal growth factor (EGF), which is known to induce cell motility and migration. Keratinocytes stimulated with EGF exhibited a 2.6-fold increase in their migration velocity, which coincided with enhanced ruffle retraction velocity (144%) and increased ruffle frequency (135%) compared to control cells. We also recorded an enhanced frequency of lamellipodia (135%), whereas the velocity of lamellipodia protrusion remained unchanged. These results on ruffle and lamellipodia dynamics in epidermal cells show that SACED is at least equal to established methods in terms of accuracy. SACED is, however, advantageous concerning resolution in time and therefore allows one to analyze the activity of lamellipodia and ruffles in as yet unknown detail. Moreover, SACED offers two opportunities that render this technique superior to established methods: First, several parameters relevant to cell motility can be analyzed simultaneously. Second, a large number of cells can conveniently be examined, which facilitates the compilation of statistically significant data. © 1999 Academic Press

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INTRODUCTION

Most eukaryotic cells are permanently, intermittently, or at certain stages of their life cycle able to move. Cell motility comprises a sequence of events involving cytoskeletal reorganization, lamella extension, substratum adhesion at the leading edge, contraction to move the cell forward, and detachment of the trailing tail [1–5; for reviews see 6, 7].

Dynamic structures observed in motile cells in culture are defined as filopodia, lamellipodia, or ruffles [8–10]. These structures occur at the cell lamella, a cytoplasmic region that borders the organelle-containing main cell body. Filopodia are spike-like protrusions that radiate from the tip of the lamella, whereas lamellipodia are flat, sheet-like structures that protrude from the lamella margin. After their protrusion lamellipodia are retracted toward the main cell body. Retraction of lamellipodia is frequently accompanied by the formation of ruffles, which are actin-containing folds rising above the lamella surface [8, 11; for review see 12]. Ruffles start at the cell edge, move centripetally, and finally disappear at the border between the lamella base and the main cell body. Centripetal movement is the main feature that distinguishes ruffles from other, immobile or centrifugally moving cell membrane structures [8]. The sequence of cell membrane protrusions and ruffle retraction results in a regular periodic oscillation of these cell membrane structures [13–15]. Formation of lamellipodia and formation of ruffles are important and likely rate-limiting events occurring during cell motility.

To elucidate the mechanisms underlying cell motility, the quantitative analysis of cell migration and of lamella dynamics, including lamellipodia and ruffle formation, is important [for review see 16]. Several methods have been developed that allow such quantitation, such as Boyden chamber assays [17], phagokinetic tracks [18, 19], artificial wound scratches [20], spheroid outmigration [21], and gel invasion assays [22]. Only few assays, however, such as computer-assisted on-line tracking methods [23–27], allow one to quantify changes in cell migration instantaneously. A

major step in analyzing cell motility was made by applying computer-assisted tools to quantify cell perimeter dynamics either of amoeboid cells [28] or of flattened tissue cells [13, 27, 29]. However, no systems were available to satisfactorily quantify subcellular dynamics at high resolution. In most cases instant changes in cell motility are qualitatively described as “enhanced ruffling and lamellipodia activity” or semi-quantitatively expressed as the “number of ruffling cells” [30–34]. Recently, a pol-scope technique was reported, which allows one to study the dynamic behavior of the cytoskeleton in the lamellipodia of growth cones using polarized light [35]. This technique provides useful information on the dynamics of cytoskeleton components, but it is not designed to quantitate cell motility.

We have developed a new tool, which consists of a computer-assisted stroboscopic analysis of cell dynamics (SACED). This technique is suitable for quantifying lamellipodia protrusion and ruffle retraction simultaneously with cell migration velocity. SACED allows subcellular movements to be revealed at defined regions of the cell membrane on the order of seconds and for a large number of cells. The technique proved to be generally applicable to any kind of microscopy of living cells in culture and was implemented on phase-contrast observations in this study.

Keratinocyte migration and the development of filopodia, lamellipodia, and ruffles are regulated by external factors like epidermal growth factor (EGF) [36–38] and transforming growth factor α (TGF α) [39–41]. These factors act as “motogens” [for review see 42] that stimulate keratinocyte migration and lamella dynamics. To demonstrate the potential of SACED we stimulated human keratinocytes with EGF and compared our results with data previously obtained by other techniques. With SACED we quantitated the effect of EGF on lamellipodia protrusions, ruffle retraction, and cell translocation. Our measurements show agreement with previously reported observations. Our observations, however, show also that the regulation of motility and migration of eukaryotic cells can now be analyzed quantitatively with high precision and at high resolution in space and in time.

MATERIALS AND METHODS

Cell culture. Cultures of human epidermal keratinocytes (HEK) were obtained from newborn foreskins and initiated into culture by modification of a method by Rheinwald [43] and Green *et al.* [44]. Subcultures were grown in MCDB 153 medium supplemented with bovine pituitary extract (240 μ g/ml), epidermal growth factor (0.1 ng/ml), ethanolamine (0.1 mM), insulin (750 μ g/ml), penicillin (100 U/ml), phosphoethanolamine (0.1 mM), and streptomycin (100 U/ml). Medium was further supplemented with the following essential amino acids: glutamine (4 mM), histidine (240 μ M), methionine (90 μ M), phenylalanine (90 μ M), tryptophane (45 μ M), and tyrosine (75

μ M). Supplemented MCDB containing 0.03 mM calcium was taken as control medium. Cells were generally used from third passage.

Wound scratch assay. HEK were grown to confluence on 8-well glass slides (ICN Biomedicals, Eschwege, Germany). Artificial wounds were scratched into the monolayer with a sterile 100- μ l pipette plastic tip. The medium was then changed twice to remove cellular debris before cells were again cultivated in different media containing or lacking EGF (see below). Media were replenished every hour. Migration of keratinocytes into the artificial wound was visually controlled and pictures were taken 15 h after scratching.

Microscopy of living cells. Microscopy was performed using a 63×1.4 NA phase plan apochromat objective on an Axiovert 10 inverted microscope (Zeiss, Jena, Germany), equipped with a video system containing a low-light video camera (AVT Horn BC-5 with control unit, Aalen, Germany) and a time-lapse videocassette recorder (Panasonic AG 6720 A). Keratinocytes from third passage were seeded at 5000 cells/cm² in observation chambers constructed of a 35-mm-diameter silicone rubber ring mounted on a 50×80 mm glass coverslip. Cells were then grown for 18 h in control medium to 50% subconfluence before experiments started. Temperature was held constant at 37°C by keeping the microscope in a ventilated incubation chamber.

Computer-assisted stroboscopic analysis of cell dynamics (SACED)—Characterization of lamella dynamics with high resolution. Phase-contrast images of motile keratinocytes were digitized using a video frame grabber card (OFG 768 PAL) and analyzed by computer-assisted SACED. SACED is based on programmable image processing software (BioScan OPTIMAS). In order to monitor dynamics of one cellular region an area of interest in the shape of a line was marked on the phase-contrast image of isolated keratinocytes. This line extended from inside the cell body ($x = 0$) to the substrate outside the cell ($x = 20$) by crossing the lamella region transversally to the cell edge. Distinct gray values of structures visible in phase contrast and included in the area of interest were then digitally recorded. By the digitizing process a snapshot of the included cell region with a width of one pixel was obtained. The position of the cell edge, of dark lamella structures, of the main cell body, and of the nucleus could be identified on this segment when compared to the original cell image (see Results).

Dynamics of this particular cell region were then visualized by recording gray values included in the area of interest at regular time intervals while the cell edge was protruding and retracting. It should be pointed out that SACED recorded lamella dynamics relative to the substrate since the position of the area of interest (the line) was fixed. In the next processing step the digital snapshots were lined up on a time scale in sequence of their acquisition. The resulting composite phase-contrast picture allowed the translocation of the recorded structures to be continuously followed over time. We named the composite picture a “stroboscopic image” since it resembled a series of flashlight snapshots. The entire process resulting in a stroboscopic image was automated by a computer program.

Quantification of cell motion by SACED with statistical significance. To quantify lamella dynamics stroboscopic images obtained from SACED recording were displayed on a computer screen. In a second computer routine structures such as protruding lamellipodia and retracting ruffles were manually labeled with lines by using a computer mouse. Since space and time were defined in stroboscopic images (20 μ m in 5 min), a number of values were automatically deducible from the label lines.

First, the computer algorithm identified a descending label line as a labeled ruffle and an ascending label line as a labeled protruding cell edge. Second, the values of motility parameters were extracted from the label lines and saved to a file that was analyzed by statistical software. Characteristic parameters of protruding lamellipodia and retracting ruffles were their velocity (micrometers per minute), their persistence (minutes), ruffle migration distance over the lamella (micrometers), extension of lamellipodia at the lamella tip

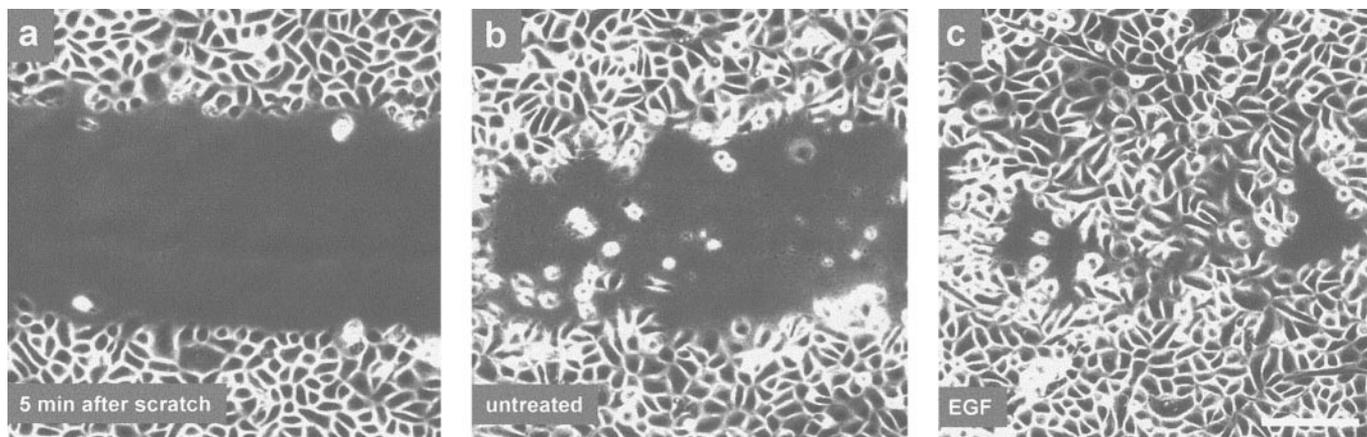


FIG. 1. Effect of EGF on scratch wound closure. In order to examine the effect of EGF on keratinocyte migration, artificial scratches were produced in a monolayer of confluent cells (a). The migration of keratinocytes into the wound gap was documented 15 h after scratching (b and c). Note that treatment with 100 ng/ml EGF (c) induced earlier wound closures compared to untreated cells (b). Bar, 100 μm .

(micrometers), the frequency of lamellipodia (lamellipodia per minute), and ruffle frequency (ruffles per minute). The migration velocity of cells (micrometers per minute) was simultaneously quantified by exploiting the clear halo around the main cell body and the nucleus. Marking this outstanding halo allowed one to calculate the cell body displacement in relation to the substrate, which is a common definition for cell translocation [28]. The visible border of the cell body was also used to calculate the minimum and maximum extensions of lamellae (micrometers).

To cover the whole cell periphery we increased the number of SACED lines crossing the lamella region of keratinocytes. Using a radial arrangement of section lines allowed changes in the leading edge position to be followed over time. SACED allowed the number of lines (areas of interest) per cell as well as their position, starting points inside the cell, their length, and arrangement, to be chosen. Eight lines of 20 μm length covered the lamella periphery of a motile keratinocyte. A radial arrangement of lines was chosen since these cells mostly exhibited a circular spreading area. In order to evaluate the motility state of cells a standard recording time of 5 min per cell was adequate. The computerized drawing of the lines was initiated first by marking the cells centroid on the computer screen and second by indicating the cells' orientation (position of the dominant lamella). Subsequently, all lines were automatically calculated and displayed. The SACED recording process resulted in eight stroboscopic images per cell that were separately displayed to quantify lamella dynamics as described above.

For quantification by SACED, lamella dynamics were recorded on eight regions at 15 different isolated cells for 5 min per cell at 37°C. Cells were allowed to equilibrate under modified conditions for 15 min. Mean values were calculated from the values extracted at all eight regions of the 15 cells per experimental condition. Results were expressed as the percentage of mean control values that were obtained from 15 untreated cells for each experimental series. To exclude aging effects, untreated control cells were recorded before and after each experiment. In order to quantify the changes in cell motility and cell migration following tyrosine kinase stimulation we applied EGF at concentrations 0, 5, 10, 25, 50, 75, 100, and 200 ng/ml. All experiments were performed three times.

Statistical analysis. For statistical evaluation, the results were analyzed by means of Student's *t* test. Differences from control cells were considered to have a high statistical significance at values of $P \leq 0.01$.

RESULTS

To demonstrate the effect of stimulation of keratinocyte migration by EGF by a classical approach we used scratch assays. Fifteen hours after an artificial wound was scratched into a confluent monolayer of keratinocytes (Fig. 1a), untreated cells had insufficiently covered the wound by migration (Fig. 1b), whereas EGF-stimulated cells had nearly closed the wound by migrating into the gap (Fig. 1c).

To test the SACED, migration and lamella activity, such as lamellipodia and ruffle formation, of keratinocytes during scratch wound closure were analyzed. In addition, the morphology of normal, unstimulated keratinocytes (Fig. 2a) was characterized on phase-contrast micrographs. In order to analyze morphological structures by SACED, a cellular region was labeled by a line (Fig. 2a, black line; Fig. 2b, frame) that extended from the cell body (Fig. 2a, dashed black line) to a cell-free region including the leading edge of the keratinocyte (Fig. 2b, arrowhead). The morphological structures present in this area of interest (Fig. 2b, frame) corresponded to pixel gray values (Fig. 2c) that were digitized. In the region of the cell body (0–7 μm), large grayscale differences ranging between light gray and dark gray values were observed. In contrast, the lamella (7–15 μm) showed medium-range gray values. The border between cell and culture medium (Fig. 2c, arrowhead) was defined by a characteristic sequence of gray values, beginning with dark gray (lamella/lamellipodia edge) followed by light gray values and medium gray values representing the substrate.

Lamella dynamics were analyzed in a series of images acquired every 0.5 s. In every picture the area

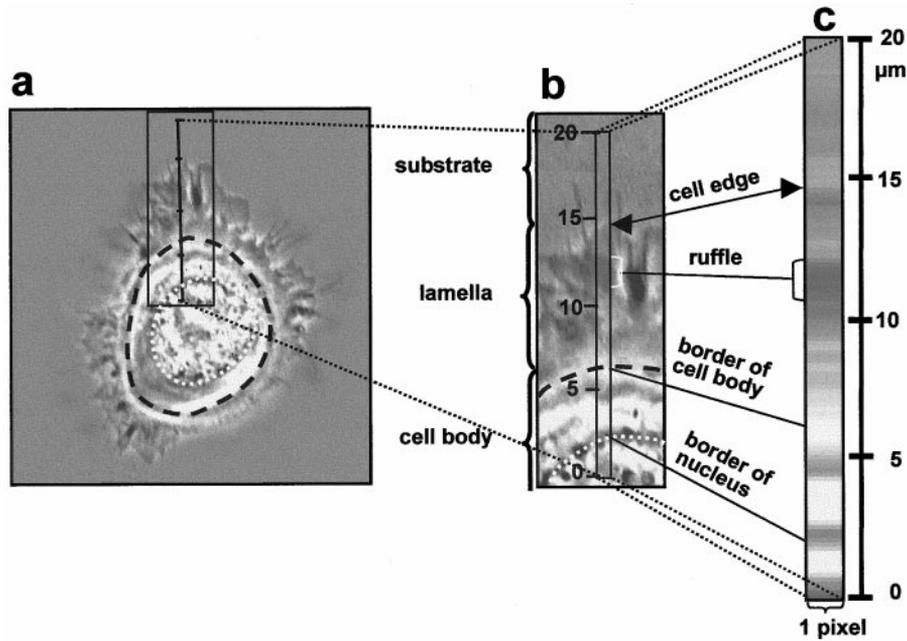


FIG. 2. The SAGED method was initiated by placing a line of defined length (here $20\ \mu\text{m}$) over the phase-contrast image of a motile keratinocyte (a). This line extended from inside the cell body ($0\ \mu\text{m}$) to the substrate ($20\ \mu\text{m}$) by crossing the lamella transversally to the cell edge. Subdivisions are displayed on the line at distances of $5\ \mu\text{m}$. A dotted white line marks the border of the nucleus, whereas a dashed black line indicates the border between the cell body and the lamella. The area of interest marked by the line is magnified in (b) and displayed as a frame. Gray values of structures included by the frame were digitized (c). Originally, the digitized line (=area of interest) had a width of 1 pixel but was here artificially broadened for the purpose of better visualization. By correlating gray values on the digitized area of interest (c) with gray values in the phase-contrast image (b), cell structures were identified as shown in Fig. 3. The position of the cell edge is indicated by arrows in (b) and (c). Ruffles are marked by brackets.

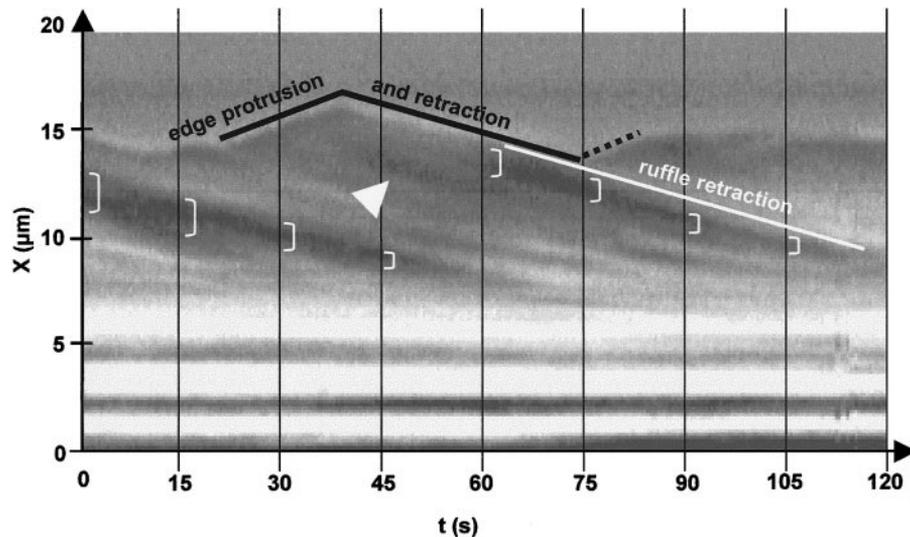


FIG. 3. Visualization of lamella dynamics by SAGED. To visualize the dynamics of gray values included by the frame in Fig. 2, the area of interest was digitized every 0.5 s. The resulting digital segments were then lined up on a time scale producing a so-defined stroboscopic image. The segments were here reduced to the original width of the line (=area of interest) of one pixel. Moving gray values corresponded to dynamic cell structures as described under Results. White brackets include retrograde moving ruffles, also indicated by a white line. Black lines were used to indicate the protruding and retracting cell edge. The arrowhead points to a ruffle not originating from a retracting lamellipodium but forming during the process of lamellipodia protrusion.

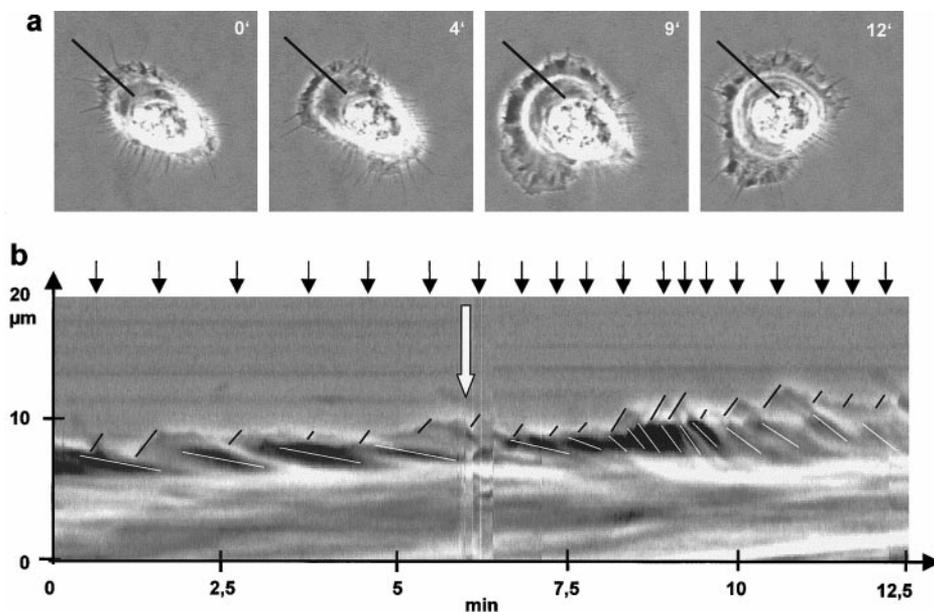


FIG. 4. Effect of EGF on lamella dynamics. To analyze the immediate effect of EGF on keratinocyte motility, we recorded lamella dynamics by 8-line SACED during cell stimulation. The period of measurement was 12.5 min. Phase-contrast images of the motile cell were produced during recording (a) as a control of the stroboscopic images (b). Only one of eight stroboscopic images is demonstrated here, recorded along the boldface line in (a). Cells were first recorded for 6 min in control medium before EGF concentration was shifted to 100 ng/ml (white arrow). Changing the medium produced disturbances in the stroboscopic image that left the plane of focus. Descending white lines label ruffle retraction, and ascending black lines indicate lamellipodia protrusion. The descent or ascent of the lines corresponded to the velocity of the respective lamella structure. Black arrows on top of the stroboscopic image indicate formation of lamellipodia.

of interest labeled by a line was digitized. The arrangement of the digitized area of interest in a sequence over time produced an image that we termed stroboscopic image according to the fast acquisition of data (Fig. 3). Movements of dynamic structures like lamellipodia, ruffles, or the cell edge corresponded to changes in the location of gray values on the stroboscopic image. Stroboscopic images were subdivided into three zones (see above): The first zone ranging from 0 to 7 μm corresponding to the cell body showed stake gray values that remained in identical locations over time. The second zone, i.e., the lamella, showed variable extensions over time ranging from 7–13 μm at 7 s to 7–17 μm at 38 s. In this zone darker gray values moved continuously from the cell edge toward the cell body. The dark gray regions had a width of 0.5–2 μm . These structures corresponded to ruffle movement over the observed period as evaluated by time-lapse video observation. Generally, ruffle movement was reproducibly identified on stroboscopic images of keratinocytes by three features (Fig. 3): (i) their dark appearance in phase contrast (low gray values), allowing a clear separation from other structures at the lamella; (ii) their characteristic maximal width of 2 μm ; and most important (iii) their unmistakable centripetal movement, starting at the cell edge and ending at the border of the cell body. In contrast, the pixel gray values within the third zone, which represented the culture medium,

remained unchanged. The border between the second and the third zones was characterized by an undulating line over time (Fig. 3). This bright undulating line corresponded to extending and retracting lamellipodia at the lamella tip of the keratinocyte, bordering the culture medium. The beginning of a lamellipodia protrusion in stroboscopic images was marked by turning points where the previously declining edge started to increase again (Fig. 3). Correspondingly, the ending of lamellipodia protrusion and the beginning of retraction were localized at turning points where the line started to decline again.

As the SACED technique revealed, untreated cells formed and subsequently retracted a lamellipodium at the lamella tip within 60 s (labeled by black lines in Fig. 3). Lamellipodia retraction led to the formation and movement of a ruffle (labeled by a white line and brackets) starting at the lamella edge. Ruffles continued the retrograde movement of the lamellipodium with identical velocity. During ruffle retraction (60–120 s) a new lamellipodium formed (75 s, dotted black line).

Visualization of Instant Changes in Lamella Dynamics by SACED after Stimulation with EGF

To demonstrate immediate changes in lamella dynamics following EGF stimulation, isolated keratino-

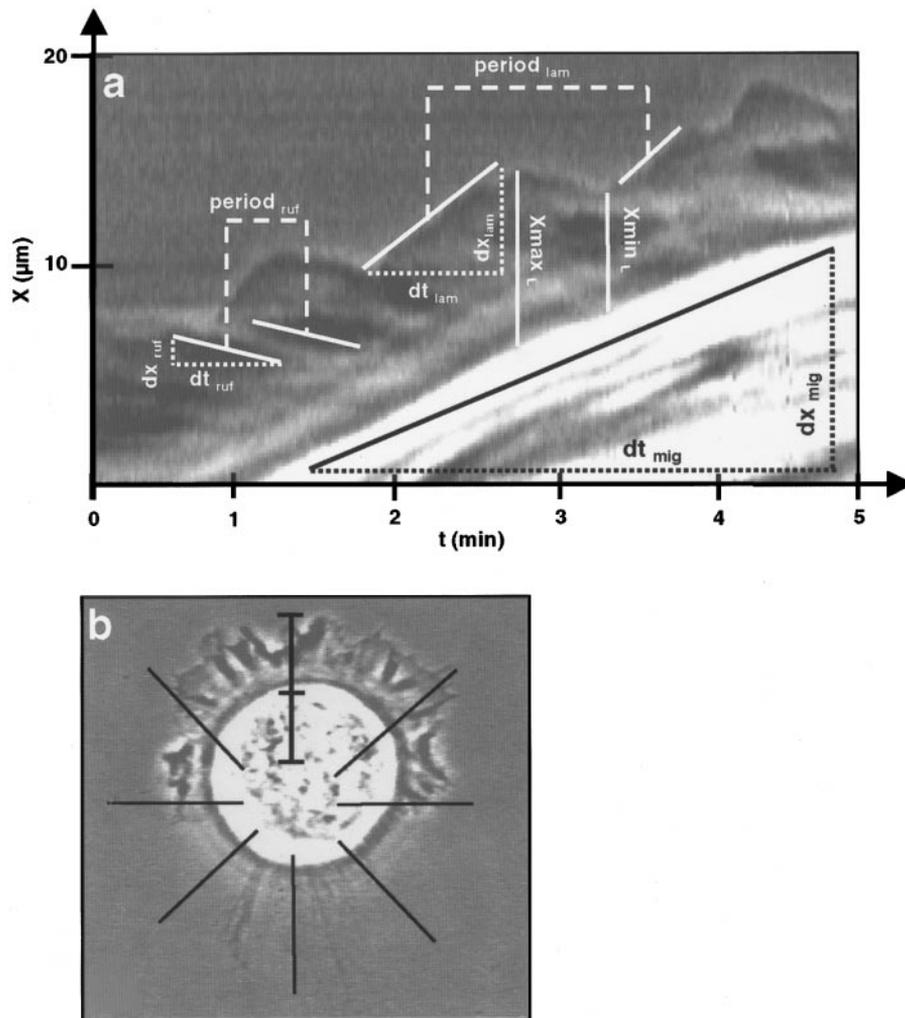


FIG. 5. Quantitation of keratinocyte motion. (a) In order to quantify cell motion, the course of retracting ruffles, protruding lamellipodia, and the translocating cell body were labeled on the stroboscopic images (continuous label lines). Along the label lines, characteristic values were calculated for ruffle retraction velocity (dx_{ruf}/dt_{ruf} , $\mu\text{m}/\text{min}$), lamellipodia protrusion velocity (dx_{lam}/dt_{lam} , $\mu\text{m}/\text{min}$), ruffle migration distance (dx_{ruf} , μm), lamellipodia extension (dx_{lam} , μm), ruffle frequency ($1/\text{period}_{ruf}$, ruf/min), lamellipodia frequency ($1/\text{period}_{lam}$, lam/min), maximum lamella extension ($X_{max,L}$, μm), minimum lamella extension ($X_{min,L}$, μm), and cell migration velocity, indicated by the cell body translocation over time (dx_{mig}/dt_{mig} , $\mu\text{m}/\text{min}$). (b) Lamella dynamics were simultaneously analyzed around the whole cell periphery of a single keratinocyte by placing eight lines of 20 μm in a radial arrangement.

cytes were assayed. Eight cell areas were marked by lines of 20 μm length, which included the lamella and the cell edge, and were recorded simultaneously by SACED for 12.5 min. First, keratinocyte motility and lamella dynamics were digitized at the areas of interest in control medium for 6 min (Fig. 4, shown for only one selected region). The switch to a medium containing 100 ng/ml EGF at 6 min induced an increase in the number of lamellipodia protrusions at the cell edge as observed by time-lapse video (Fig. 4b). These changes were reflected in the digitized area of interest (line in Fig. 4a; Fig. 4b). Following EGF stimulation (white arrow in Fig. 4b) cells enhanced the frequency of lamellipodia (number of black label lines per time)

after a lag phase of 2 min, whereas the velocity of lamellipodia protrusion (ascent of black label lines) was not altered. Ruffle formation (white lines) as well as ruffle retraction velocity increased. Immediately after EGF stimulation (7–10 min) lamella dynamics accelerated but regained a periodic behavior after the acceleration phase (>10 min). Periodicity was most evident from the formation of ruffles with similar retraction velocity in regular time intervals. To exclude the possibility that the observed effects were caused by changing the medium, we also performed a series of experiments shifting cells to BSA-containing medium. In neither case was a change in ruffle and lamellipodia dynamics observed.

TABLE 1
Mean Values of Human Keratinocyte Motion
Obtained from SACED

	Control	EGF
Migration velocity (dx_{mig}/dt_{mig} ; $\mu\text{m}/\text{min}$)	0.5 ± 0.3	1.3 ± 0.4
Ruffle retraction velocity (dx_{ruf}/dt_{ruf} ; $\mu\text{m}/\text{min}$)	4.1 ± 1.3	6.0 ± 2.0
Lamellipodia protrusion velocity (dx_{lam}/dt_{lam} ; $\mu\text{m}/\text{min}$)	7.5 ± 2.5	7.3 ± 2.8
Ruffle frequency ($1/\text{period}_{ruf}$; ruf/min)	2.4 ± 1.5	3.2 ± 1.8
Lamellipodia frequency ($1/\text{period}_{lam}$; lam/min)	1.3 ± 0.8	1.8 ± 0.9

Note. We here show absolute values for a selection of typical parameters that express the motility of tissue cells jointly with cell migration velocities. Mean values and standard deviations were calculated from 8-line SACED. Values are compared among 100 cells grown in control medium with 15 cells that were subsequently stimulated and recorded for maximum 1 h in medium containing 100 ng/ml EGF.

SACED Quantified Changes in Cell Motion Following EGF Stimulation

To determine the mean values of typical motility parameters, lamella dynamics in keratinocytes were quantified on a single-cell scale by applying SACED to

keratinocytes in the absence of EGF (Fig. 5a). The parameters measured included the following: ruffle retraction velocity (dx_{ruf}/dt_{ruf} ; micrometers per minute), lamellipodia protrusion velocity (dx_{lam}/dt_{lam} ; micrometers per minute), ruffle migration distance (dx_{ruf} ; micrometers), lamellipodia extension (dx_{lam} ; micrometers), ruffle frequency ($1/\text{period}_{ruf}$; ruffles per minute), lamellipodia frequency ($1/\text{period}_{lam}$; lamellipodia per minute), maximum lamella extension (X_{max_L} ; micrometers), minimum lamella extension (X_{min_L} ; micrometers), and the cell migration velocity (dx_{mig}/dt_{mig} ; micrometers per minute). Mean values, obtained from 100 cells, were calculated from eight areas of interest per cell. The radial arrangement of these eight areas is illustrated in Fig. 5b. In untreated keratinocytes, lamellipodia protrusion was found to be much faster ($7.5 \pm 2.5 \mu\text{m}/\text{min}$) than ruffle retraction ($4.1 \pm 1.3 \mu\text{m}/\text{min}$). Conversely, ruffle frequency was about two times higher (2.4 ± 1.5 ruffles/min) than lamellipodia frequency (1.3 ± 0.8 lamellipodia/min). These results were in line with the observation from time-lapse recordings that ruffle formation may not only occur during lamellipodia retraction but may also be initiated during lamellipodia extension (arrowhead in Fig. 3).

Keratinocytes showed characteristic and significant changes in lamella dynamics and cell migration depending on the presence of EGF. Migration velocity,

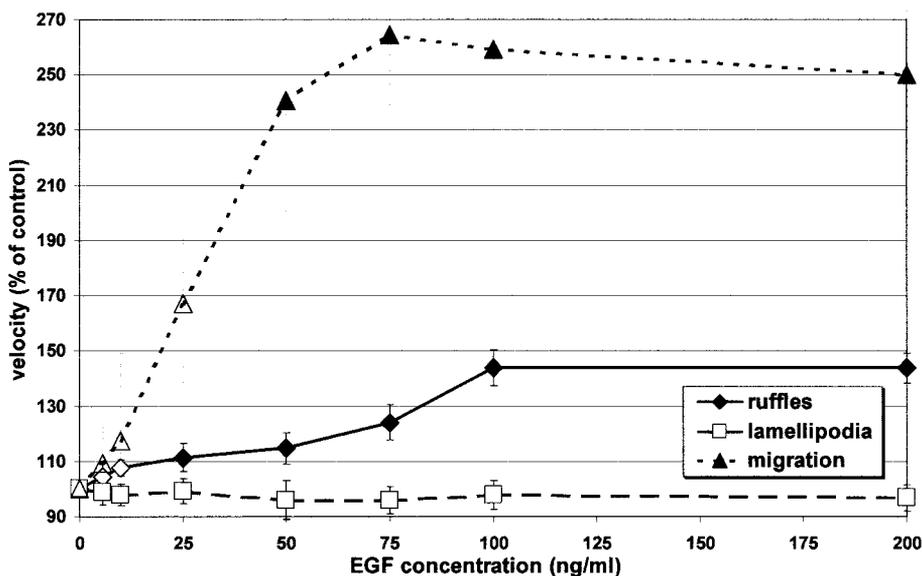


FIG. 6. Quantitation of EGF-stimulated keratinocyte motility. Human keratinocyte motility was stimulated by EGF in a concentration-course experiment. Before EGF stimulation cells were grown for 18 h in control medium, followed by stimulation with EGF at concentrations of 5, 10, 25, 50, 75, 100, and 200 ng/ml, 15 min before SACED analysis. For each concentration eight areas of interest of 15 different cells were analyzed. To exclude aging effects control cells were recorded at the beginning and at the end of each experiment. Mean values of ruffle retraction velocity (continuous line), lamellipodia protrusion velocity (disconnected line), and migration velocity (dotted line) were calculated as percentage of the respective control mean values. Experiments were performed three times; error bars indicate 95% confidence intervals. Values that deviate from the controls with high significance ($P \leq 0.01$) are depicted as black symbols. White symbols refer to values that do not differ significantly from control values.

ruffle retraction velocity, ruffle frequency, and lamellipodia frequency increased after stimulation with EGF, whereas lamellipodia protrusion velocity remained constant (Table 1). SACED offered the possibility of demonstrating these changes in concentration-course experiments (Fig. 6). Mean values were calculated and plotted as percentage of controls. With increasing EGF concentration, ruffle retraction velocity was gradually enhanced to maximum of 144% of controls at 100 ng/ml EGF (Fig. 6). This correlated with a significantly enhanced migration velocity, reaching 255% of the control at 100 ng/ml EGF. In contrast, the velocity of lamellipodia protrusion did not change significantly even at high EGF concentrations. Both lamellipodia frequency and ruffle frequency were enhanced to a maximum of 135% by 100 ng/ml EGF (not shown).

DISCUSSION

The aim of this study was to characterize the motion of keratinocytes by a new technique consisting of computer-assisted stroboscopic analysis of cell dynamics, SACED. This method is a major improvement in that it creates the possibility of quantitatively analyzing the motion of cells by characterizing simultaneously the dynamics of lamellae, including lamellipodia and ruffle formation, of cultured cells with high resolution in space and in time. Changes in these dynamics can be investigated in defined regions of one cell. Owing to the high rate of data acquisition, a statistically significant number of cells can reliably be examined by SACED.

Keratinocyte migration is usually evaluated by cell migration assays such as wound scratch assays, colony dispersion assays, or phagokinetic assays [37–39, 45–48]. A number of previous studies allowed quantitative data on ruffle and lamellipodia dynamics in epidermal cells [49], fibroblasts [8, 11, 14, 50, 51], or neuronal growth cones [52] to be acquired. In contrast to SACED, however, the methods used in these studies involved time-consuming manual analyses of lamella dynamics with only limited resolution in time. Moreover, time-consuming manual measurements limited the number of cells to be examined.

According to these studies ruffle retraction velocity in cultured tissue cells was determined in the range of 3–5 $\mu\text{m}/\text{min}$. Lamellipodia extension velocity was observed to range from 2 to 8 $\mu\text{m}/\text{min}$. The magnitude of the observed velocities corresponds to our quantification of keratinocyte dynamics. Until now, the velocity of ruffle retraction or lamellipodia protrusion in human keratinocytes has not been documented.

The effect of EGF on cell migration has been analyzed in human keratinocytes. Using a phagokinetic assay Chen *et al.* [53] reported an increase in keratinocyte migration after 1 ng/ml EGF stimulation from 1.7- to 6.3-fold after 16 h, depending on the substrate.

In fibroblasts, stimulation with 25 μM EGF increased cell migration rates to 60% after 24 h as measured by scatter assays [36]. On the other hand, wound scratch assays reported an increase of cell migration of 200% [36] and of 220% [54] after 24 h. In terms of absolute single-cell velocity recordings only few data are available regarding EGF stimulation. In a recent publication, a sophisticated computer analysis was used to demonstrate a maximal 3.5-fold increase of fibroblast migration velocity from 0.5–0.7 to 0.5–1.5 $\mu\text{m}/\text{min}$ 8 h after EGF stimulation depending on substrate density [27].

However, these studies did not address quantitative aspects of EGF stimulation on lamella dynamics, especially on a short-term scale. At present, EGF has only qualitatively been reported to possess stimulatory effects on cultured cell membrane ruffling [31, 55] and lamellipodia extension [56]. In this study we have applied SACED to analyze the instant increase of lamella dynamics after shifting cells to high EGF concentrations. The novel observations indicate that lamellipodia protrusion and ruffle retraction may be separately regulated, since only ruffle retraction velocity is enhanced, whereas lamellipodia protrusion velocity remains unaffected. Increased lamella and ruffle retraction is presumably compensated for by an enhanced frequency of lamellipodia protrusion formation.

Preliminary results with AG1478, an inhibitor of EGFR kinase, confirmed that EGF exerts a differential effect on lamella dynamics. Application of this inhibitor predominantly reduced ruffle retraction velocity, whereas the velocity of lamellipodia protrusion was affected to only a minor extent (unpublished results).

It is generally agreed that lamella dynamics are an important prerequisite for cell migration [7, 10, 11, 26, 57–60]. In this study we have shown that the EGF-induced cell migration is associated with increased ruffle retraction velocity. Hence, our observations show that ruffle retraction velocity and the frequency of lamellipodia formation have a major regulatory influence on promoting cell migration, whereas the velocity of lamellipodia protrusion is of minor importance.

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