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Quantitative Measurement of Cell Motility Associated with Transformed Phenotype

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The motility of individual mammalian cells is crucial for many biological processes. This report describes a new technique to quantitate cell motility, momentary alterations of cell shape, based on trace images obtained by video-image analyses and computer techniques. By means of this system, quantitation of cell motility could be automatically done without human observation or subjective judgement. Quantitative data from transformed and nontransformed rodent fibroblasts revealed that the cell motility measured here was related to the expression of such transformed phenotypes as morphological changes and tumorigenicity.

Key words: Cell motility — Cell orientation — Video microscopy — Contrast enhancement — Digital image processing

Many biological processes such as wound healing, embryogenesis, immune system response, and cancer metastasis require the motility of individual cells.[1,2] Various techniques for measuring cell motility are now available.[3,4] In general, these techniques can be divided into two types, direct and indirect.[3,4] The latter includes micropore filter assay, agarose assay, and colloidal gold assay, but they do not examine individual cells which are actually moving. The former includes time-lapse cinematography and videomicroscopy, optoelectronic methods, and video-image analysis. These systems give detailed information on cell movement, but as yet it has been difficult to quantitate cell behavior such as cell orientation and alterations of cell shape except for approaches which depend upon human observation and subjective judgement.[3-6] We describe here a new method that can eliminate human judgement and can objectively, conveniently and precisely quantitate cell behavior.

In our system, cell behavior was recorded by a video tape recorder. The quantitative estimation of cell motility was done by counting the total intensity of the cumulative trace image in a window on a TV monitor. A trace image was obtained by subtracting a digital image for cells in any one video frame from a digital image for the same cells in the subsequent frame, and trace images for these particular cells were accumulated at intervals corresponding to the video frames (cumulative trace image). In this system, video images obtained by phase-contrast microscopy were not useful for quantitation because of different intensities of cell images between transformed and nontransformed cells in a video frame (Fig. 1-a). Using video images obtained by the Allen video-enhanced contrast-differential interference contrast (AVEC-DIC) microscopy,[7,8] it became possible to quantitate cell motility, since there is no difference in cell image intensity between transformed and nontransformed cells (Fig. 1-b). Fig. 1-c shows the trace image obtained by accumulating four video frames at 20 s intervals using the AVEC-DIC microscopic video images. The intensity of the trace image is higher in transformed cells than in nontransformed cells (Fig. 1-c).

The quantitative value of cell motility was defined as follows. $D(x, y)$ is a digital image for any one pixel (eight bits resolution) having x, y-coordinates in a matrix consisting of 640×480 pixels. For any one video frame (n) and its subsequent frame ($n+1$), a trace image, $\Delta D(x, y)$, is given as:

$$\Delta D(x, y)=D(x, y)_{n+1}-D(x, y)_{n},$$

which is positive or is zero when $\Delta D(x, y)<0$. The intensity of a trace image in a window (consisting of 100×100 pixels) for any one video frame (In) is

$$\mathrm{In}=\sum_{x=1}^{100}\sum_{y=1}^{100}\Delta D(x, y),$$

and the result of accumulation (intensity of cumulative trace image) "m" times is

$$\sum_{n=1}^{m}\mathrm{In}=\sum_{n=1}^{m}\sum_{x=1}^{100}\sum_{y=1}^{100}\Delta D(x, y).$$

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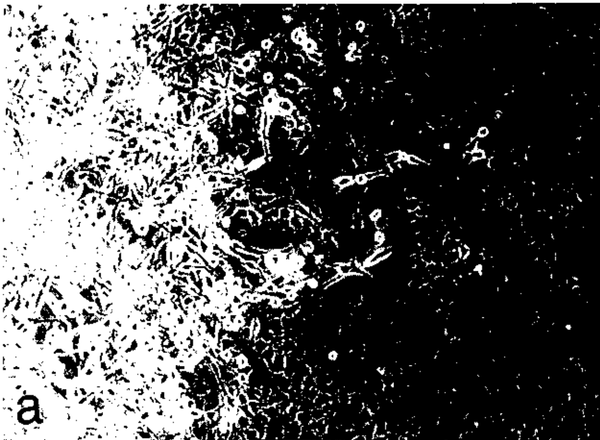
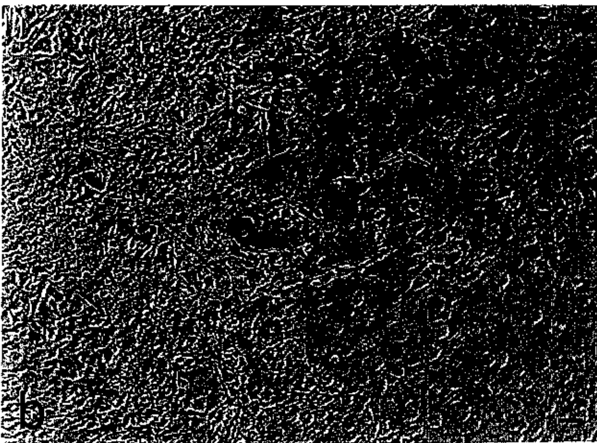
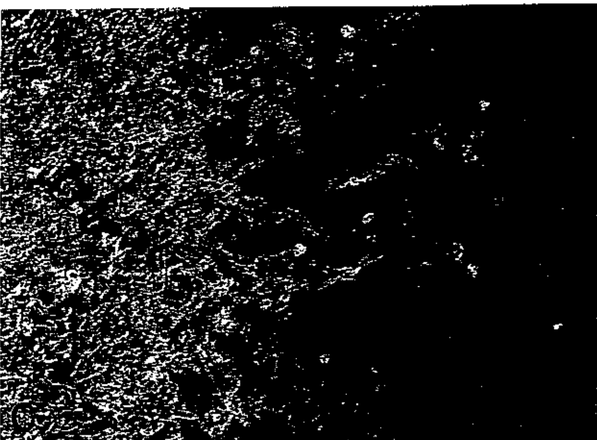
For images without cells (mainly including electric noise), the same accumulation gives:

$$\sum_{n=1}^{m} \text{In}^* = \sum_{n=1}^{m} \sum_{x=1}^{100} \sum_{y=1}^{100} \Delta D^*(x, y),$$

where In^* is an intensity of a blank trace image for any







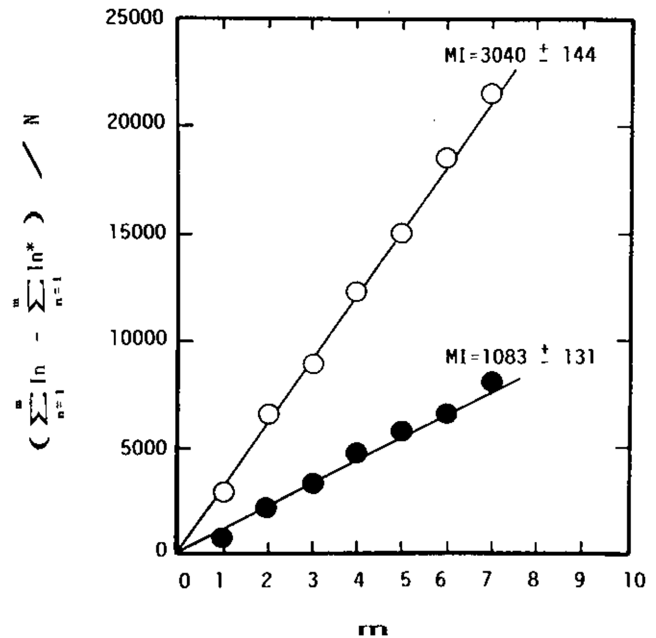


Fig. 2. Kinetics of the intensity of the cumulative trace image with respect to the number of accumulations of trace images in transformed (○) and nontransformed (●) Balb/c 3T3 A31-I-13 cells shown in Fig. 1. The formula for the ordinate is defined in the text, and the abscissa shows the number of accumulations of the trace images. The phase-contrast microscopic images or the differential interference contrast microscopic images of intact living cells at 37°C in a humidified 5% CO_2 incubator were detected by a chalnicon camera head (Hamamatsu Photonics, Hamamatsu) and transmitted to a computer-aided digital image processing unit (Photonic Microscope System Image Processor C1966, Hamamatsu Photonics). Images generated by the C1966 were recorded on U-matic video cassette tape (KCA-60KB, Victor, Tokyo) by a U-matic video cassette recorder (CR-8200, Victor), or, if necessary, by a time-lapse video system which consisted of the video recorder, a time-lapse control unit (Victor) and a light-control unit for phase-contrast microscopy (Sankei, Tokyo). Then, the recorded images on the cassette tape were transmitted again to the C1966 and analyzed by using the TRACE command for obtaining the trace images and the DATA ANALYSIS command for obtaining their intensity. Motility index (MI) was defined in terms of the kinetics of the image intensity, as described in the text.

Fig. 1. a, Phase-contrast microscopic image of a transformed focus of post-confluence Balb/c 3T3 A31-I-13 cells[9] following X-irradiation. b, AVEC-DIC microscopic image. c, Cumulative trace image (four accumulations). Each image was obtained by the method described in the legend to Fig. 2. In the image of a, the ratio of $\sum_{x=1}^{100} \sum_{y=1}^{100} D(x, y)$ for the transformed focal area to that for the area showing nontransformed morphology was 4.65, where $D(x,y)$ is a digital image for any one pixel (eight bits resolution) with x,y-coordinates in a matrix consisting of 640 × 480 pixels. In the image of b, the ratio was 1.02. Thus, the AVEC-DIC image shows no difference in intensity of the cell image between transformed cells and nontransformed cells. Scale bars, 50 μm.

Table I. MI Values of Transformed and Nontransformed Rodent Fibroblasts in the Growing State and the Post-confluence State

Cell	Agent	Nontransformed morphology: Motility index (intensity per cell per interval) Growing state	Nontransformed morphology: Motility index Post-confluence state	Nontransformed morphology: Tumorigenicity[b]	Transformed morphology: Motility index (intensity per cell per interval) Growing state	Transformed morphology: Motility index Post-confluence state	Transformed morphology: Tumorigenicity	P[a] Growing state	P[a] Post-confluence state
Balb/c 3T3 A31-I-1									
	—[c]	2488±245[d]	352±100	0/5					
	X-ray	2162±282	583±108	0/5	4351±410	1821±132	5/5	<0.05*	<0.05*
		2525±342	534±132	0/5	5306±385	1901±114	5/5		
	UV	2808±185	512±126	0/5	7273±463	2599±276	5/5		
		1994±281	643±115	0/3	4525±621	2314±177	3/3	<0.05*	<0.05*
		2718±216	550±109	0/1	4961±501	1825±163	1/1		
	B[a]P	1876±287	277±87	0/5	4447±410	1960±204	5/5		
		2224±308	486±181	0/5	4733±511	2005±188	5/5	<0.05*	<0.05*
		2719±212	860±110	0/5	5189±311	1988±149	5/5		
		2840±206	537±98	0/5	6020±530	2325±151	5/5		
	4NQO	2800±277	611±107	0/5	4361±572	1894±172	5/5		
	ras	2686[e]	457	0/5	3944	1222	5/5		
		2194	311	0/5	4506	1652	5/5	<0.05*	<0.05*
		2490	329	0/5					
	fgr	1889	219	ND[f]	4774	1985	ND		
		2616	366	ND					
NIH 3T3									
	—	2152	521	0/5					
	ras	2582	674	0/5	5546	1888	5/5	<0.05**	<0.05**
					5674	2450	5/5		
SHOK									
	—	2803	536	0/3					
	ras	2938	701	0/3	6911	2639	3/3	<0.05**	<0.05**
					5683	1940	3/3		

Radiation- or chemical-induced transformation in Balb/c 3T3 A31-I-1 cells was performed by the standard procedure described previously,[9] and cells were treated with X-ray, UV, benzo[*a*]pyrene (B[a]P) or 4-nitroquinoline-1-oxide (4NQO) at 2 Gy, 5 J/m², 0.1 μg/ml for three days or 0.1 μg/ml for three days, respectively. Oncogene-induced transformation in Balb/c 3T3 A31-I-1, NIH 3T3 and SHOK cells was performed by the electroporation method described previously,[14] and cells were transfected with 2 μg/100 μl of plasmid DNA (*ras*, pT22 plasmid containing c-Ha-*ras* gene ligated to pSV2-*neo* plasmid; *fgr*, proviral DNA of GR-FeSV ligated to pSV2-*neo* plasmid). After the formation of transformed foci, cells in focal areas or focus-like areas of each cultured dish were picked up and subsequently cultured. At the third passage (radiation- or chemical-treated cells) or at the sixth passage (oncogene-transfected cells), cells were seeded onto dishes (10^5 cells per dish) for measurement of MI values. At two days (growing state) or ten days (post-confluence state) after inoculation, MI values were measured by the method described in the legend to Fig. 2.

a) Statistical analyses were performed by use of the *t* test (Student). * Cell lines which reverted to nontransformed morphology versus cell lines which showed irreversibly transformed morphology for each agent. ** Nontransformed cell lines (control) versus transformed cell lines for each oncogene.

b) To test tumorigenicity; individual clones were washed with phosphate-buffered saline (without calcium) and injected subcutaneously into nude mice (10^6 cells per animal). No. of mice with tumors/No. of mice injected.

c) Untreated, nontransformed cells.

d) Three independent measurements were performed. Each datum is an average value±standard deviation.

e) One experiment was done.

f) Not determined.

one video frame, and $\Delta D^*(x, y)$ is positive or is zero when $\Delta D^*(x, y)<0$. Then, $(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N$ can be assumed to be a function of the video frame, where N is the number of cells in a window for $\sum_{x=1}^{100}\sum_{y=1}^{100}\Delta D(x, y)$, and is estimated visually from the phase-contrast microscopic image. Therefore,

$$(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N=f(m).$$

When the values of $(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N$ were measured in video frames and plotted against m (the number of accumulations of the trace images) (Fig. 2), $(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N$ was found to be a linear function of "m" (under the experimental conditions). So that,

$$f(m)=\alpha m,$$

where α is the constant. Then,

$$(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N=\alpha m.$$

By substitution,

$$\alpha=(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N\cdot m.$$

Since α is a constant (with "m"), we define this as the motility index (MI). Then,

$$\mathrm{MI}=(\sum_{n=1}^{m}\mathrm{In}-\sum_{n=1}^{m}\mathrm{In}^*)/N\cdot m.$$

Namely,

$$\mathrm{MI}=\Big\{\sum_{n=1}^{m}\sum_{x=1}^{100}\sum_{y=1}^{100}[D(x, y)_{n+1}-D(x, y)_n] -\sum_{n=1}^{m}\sum_{x=1}^{100}\sum_{y=1}^{100}[D^*(x, y)_{n+1}-D^*(x, y)_n]\Big\}/N\cdot m,$$

where $D(x, y)_{n+1}-D(x, y)_n$ and $D^*(x, y)_{n+1}-D^*(x, y)_n$ are positive or are zero when these values are negative. Here "m" is the number of accumulations of the trace images. We usually fix "m" at four and the time interval between video frames at 20 s (600 video frames in real time, 1/30 s per video frame). This interval (20 s) was decided on the basis of our early experiments, which were qualitatively performed by observing the time-lapse video tape images at various intervals (1/30–180 s). Thus, the MI value is defined as cell motility per cell per video frame interval.

In focus assay of chemical- or radiation-induced transformation of Balb/c 3T3 A31-I cells, the morphology of transformed foci is heterogeneous, ranging continuously from nearly nontransformed cells to markedly altered cells.[9] These foci can be categorized into two classes in terms of both morphological diagnosis and tumorigenicity when injected subcutaneously into nude mice (i.e. focal area, transformed; focus-like area, nontransformed).[9] We isolated cells from focal areas and focus-like areas in chemical or physical carcinogen-treated Balb/c 3T3 A31-I-1 cell cultures, and at the third passage after clonal expansion, cells were examined for MI values in both the growing state and the post-confluence state. The MI values of cells isolated from focal areas which showed irreversibly altered morphology were higher than those of cells isolated from focus-like areas which reverted to nontransformed morphology in both states (Table I). Similar results were obtained in oncogene-transfected cell cultures. Cells isolated from focal areas which appeared in oncogene-transfected cultures occasionally reverted to nontransformed morphology. We isolated cells from focal areas in oncogene-transfected Balb/c 3T3 A31-I-1, NIH 3T3[10] and SHOK[11,12] cell cultures, and at the sixth passage after clonal expansion, cells were examined for MI values. The MI values of stable transformed cells were higher than those of cells that reverted to nontransformed morphology (Table I). From these results, we concluded that cell motility in terms of the MI defined here and transformed phenotype (morphological alterations and tumorigenicity) are correlated with each other.

In this paper, we have described a new technique for quantitating cell motility. In this system, quantitation of cell motility is based upon the trace image which is generated by the subtraction of sequential video frames at regular intervals. This image corresponds to cell motility, including shape changes such as reffling and other motions which accompany cell locomotion (vectorial translation) and/or occur at a fixed position without vectorial translation. By means of the trace image, we found a correlation between the cell motility in terms of MI value and transformed phenotype. Although it is not clear whether movement as the result of vectorial translation is involved in the MI value, we have observed that there is no difference in cell behavior with respect to vectorial translation between two cell types, using the time-lapse video tape recorder. Other investigators also reported that no difference was observed between transformed and nontransformed mouse fibroblasts in cell movement and the speed of cell locomotion.[13] Thus, cell motions at a fixed position should be predominant for the MI value described here, and these motions might be important to express transformed phenotype. Our present work is a new approach to cancer cell biology, and may provide a powerful new tool in biological and biochemical analyses of transformed phenotype.

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