Oral Cancer Diagnosis by Mechanical Phenotyping

Torsten W. Remmerbach,[1,4] Falk Wottawah,[2] Julia Dietrich,[1] Bryan Lincoln,[2] Christian Wittekind,[3] and Jochen Guck[2,5]

[1]Department of Oral, Maxillofacial and Facial Plastic Surgery, [2]Institute of Experimental Physics I, and [3]Institute of Pathology, University of Leipzig, Leipzig, Germany; [4]Oral and Maxillofacial Surgery, School of Dentistry and Oral Health, Griffith University, Brisbane, Queensland, Australia; and [5]Department of Physics, Cavendish Laboratory, University of Cambridge, Cambridge, United Kingdom

Abstract

Oral squamous cell carcinomas are among the 10 most common cancers and have a 50% lethality rate after 5 years. Despite easy access to the oral cavity for cancer screening, the main limitations to successful treatment are uncertain prognostic criteria for (pre-)malignant lesions. Identifying a functional cellular marker may represent a significant improvement for diagnosis and treatment. Toward this goal, mechanical phenotyping of individual cells is a novel approach to detect cytoskeletal changes, which are diagnostic for malignant change. The compliance of cells from cell lines and primary samples of healthy donors and cancer patients was measured using a microfluidic optical stretcher. Cancer cells ($n \approx 30$ cells measured from each patient) were on average 3.5 times more compliant than those of healthy donors [$D_{normal} = (4.43 \pm 0.68)\ 10^{-3}\ Pa^{-1}$; $D_{cancer} = (15.8 \pm 1.5)\ 10^{-3}\ Pa^{-1}$; $P < 0.01$]. The diagnosis results of the patient samples were confirmed by standard histopathology. The generality of these findings was supported by measurements of two normal and four cancer oral epithelial cell lines. Our results indicate that mechanical phenotyping is a sensible, label-free approach for classifying cancer cells to enable broad screening of suspicious lesions in the oral cavity. It could in principle be applied to any cancer to aid conventional diagnostic procedures. [Cancer Res 2009;69(5):1728–32]

Introduction

Oral squamous cell carcinomas (OSCC) are among the 10 most common cancers in the world (1). Due to difficulties with early clinical and histologic diagnosis and lack of an unequivocal molecular marker for clinical outcome, the death rate for this particular cancer is higher than for cutaneous malignant melanomas or cervical cancer (1, 2). Despite great efforts in handling oral cancer, the prognosis for many patients is devastating with a 5-year survival rate below 50%. Earlier diagnosis is currently the most promising avenue for improving survival rates but can only be achieved by means of targeted screening measures applied on a broad scale provided that appropriate diagnostic procedures are available.

Conventional histologic investigation requires an invasive scalpel biopsy to obtain the tissue specimen of interest, cannot easily be performed as a screening measure, and is prone to subjective errors. However, this seems to be the only accepted method to evaluate suspicious oral lesions. An improved diagnostic procedure should, thus, be easily performed without surgical intervention, require only a few cells, and be based on a quantifiable physical variable to reduce operator errors.

Using the mechanical properties of single cells as a diagnostic marker could offer a sensible target for such screening. The mechanical properties of human cells are largely governed by the cytoskeleton. Although the genetic and molecular origin of cancer is multifaceted, continuous changes in the actin cytoskeleton during the progression of the disease are common to all forms of cancer (3). During the descent of the cell from a mature, differentiated state to a highly mitotic and motile cancer cell, the cytoskeleton regresses from an ordered and fairly stiff structure to a more irregular and compliant state (3, 4). Consequently, these cytoskeletal alterations should lead to a change in the mechanical fingerprint of the individual cells.

The mechanical properties of individual cells can be determined with a microfluidic optical stretcher (μOS), a dual-beam laser trap optimized to trap and deform single cells in suspension by two counterpropagating laser beams (Fig. 1; ref. 5). The measurable variable is the temporal development of the relative radial elongation of the cell, or strain (Fig. 1*D*), when subjected to a constant mechanical stress (Supplementary Fig. S1). Cells display viscoelastic behavior, following the applied stress with some retardation (Fig. 2). Using the tensile creep compliance (the strain normalized by the peak stress σ_0 applied) allows the direct comparison of cell deformability between different cells. A more compliant (i.e., more easily deformed) cell exhibits a higher D at a particular time t.

To investigate the potential of cell mechanics as a novel marker for OSCCs, we analyzed the compliance of normal and cancer cells from cell lines and, for the first time, also from primary cell samples in a clinical setting obtained from healthy and oral cancer patients. The assessment was confirmed by standard histopathology.

Materials and Methods

Compliance measurements with a μOS. Measurements were performed as described elsewhere (6). Briefly, individual cells were trapped by two laser beams at low laser power and subsequently stretched out by an elevated optical stress σ at a high laser power. The peak stress ranged from $\sigma_0 = 1$ to 5 Pa and was calculated as described previously (5). The duration of the stretch was 0.8 to 2 s. The response of the cells in the μOS was monitored via video microscopy and analyzed by custom-made algorithms.

Primary cells. Primary oral cells were obtained by tissue biopsy from the oral mucosa of four voluntary donors and five cancer patients (see Table 1).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Jochen Guck, Department of Physics, Cavendish Laboratory, University of Cambridge, Cambridge CB3 0HE, United Kingdom. Phone: 44-1223-748914; Fax: 44-1223-337000; E-mail: jg473@cam.ac.uk.

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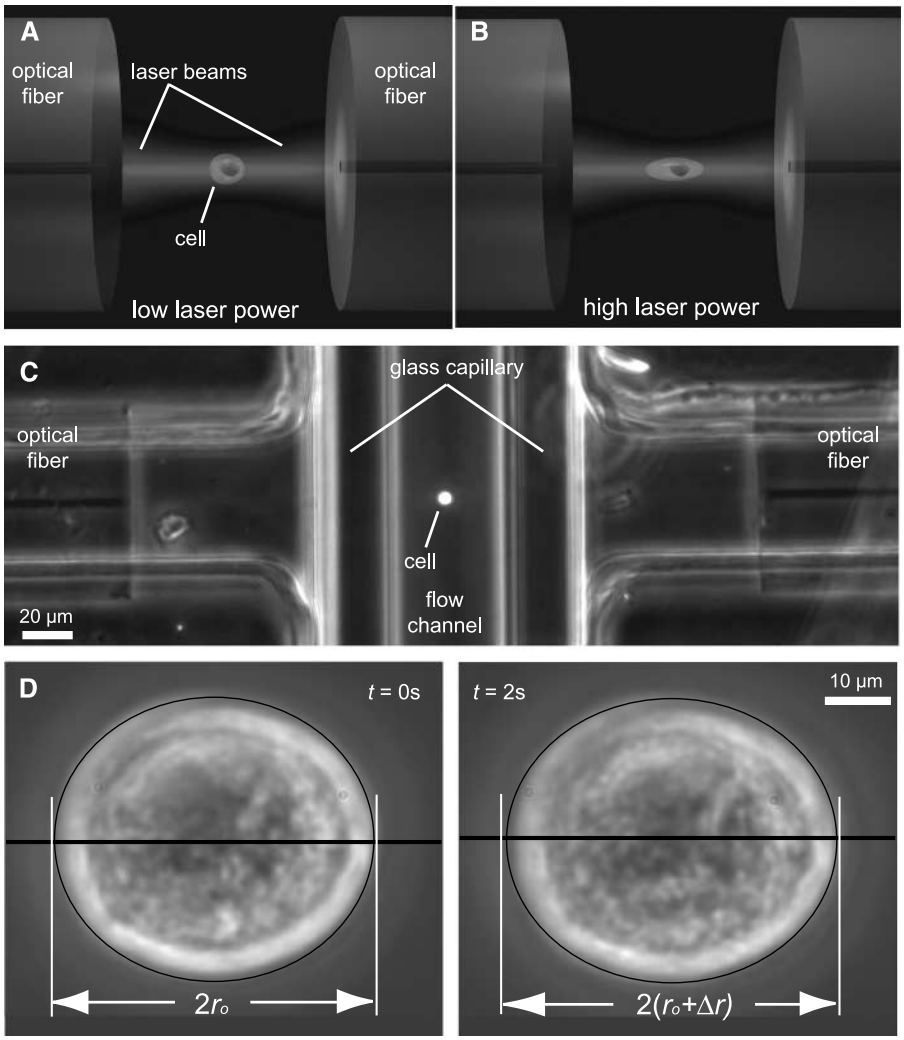

Figure 1. Working principle of the μOS. *A,* a cell is trapped between two counterpropagating divergent laser beams (λ = 1,064 nm) at low laser power (P = 0.2 W), where there is no measurable deformation. *B,* by increasing the power (P = 1.4 W), the surface stress is sufficient to deform the cell along the laser beam axis. *C,* phase-contrast image of the experimental arrangement. A cell is trapped from a flowing suspension inside a glass microcapillary by two laser beams (not visible) that emanate from opposing single-mode optical fibers. *D,* phase-contrast images of an OKF-6 cell in the μOS at the beginning (t = 0 s) and after application of elevated stress (t = 2 s). The relative radial deformation [strain, $\varepsilon(t) = \Delta r(t)/r_o$] along the laser beam axis is measured by video microscopy.

The ethics committee of the University of Leipzig approved the research protocol and all participants gave written informed consent according to the Declaration of Helsinki. All biopsies from cancer patients were confirmed histologically. Tissue samples were disinfected with Betaisodona (Mundipharma), rinsed twice in PBS, and placed in 2.5 mg/mL Dispase II (Roche) in DMEM for 18 to 24 h at 4°C. Subsequently, the epidermis was pealed off, fragmented, and incubated in 0.25% trypsin/EDTA for 5 min. Trypsin activity was stopped with DMEM containing 10% serum. The suspension was centrifuged, resuspended in keratinocyte basal medium (Clonetics) with 100 units/mL penicillin and 100 μg/mL streptomycin, and cultured at 37°C/5% CO_2. All primary samples were measured within a few days after biopsy (see Supplementary Fig. S2).

Cell lines. Normal oral epithelial cell lines OKF-4TERT1 and OKF-6TERT1 (7) and oral epithelial cancer cell lines HN (8), BHY (8), CAL-27 (9), and CAL-33 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; ref. 9) were cultured in a 37°C/5% CO_2 environment. OKF cells were cultured in keratinocyte serum-free medium (Life Technologies/Invitrogen) supplemented with epidermal growth factor (3.22 μg/mL), bovine pituitary extract (12.52 mg/mL), calcium chloride (0.3 mmol/L), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cancer cell lines were cultured in DMEM with high glucose (Sigma-Aldrich), 20% fetal bovine serum, and 5 mL HEPES. Before measurement, cells were trypsinized, centrifuged, and resuspended in medium.

Statistical analysis. Because normal distributions could not be established, a nonparametric Mann-Whitney U test was used to test for significance. The P values for a 95% confidence interval are reported.

Results

The deformability of individual normal and cancerous oral epithelial cells was serially investigated with a μOS. All cells were singularized and prepared before measurement as described above. The different viscoelastic responses of the cells under stress are shown in Fig. 2. The temporal development of the deformability displayed typical viscoelastic behavior on the timescale of seconds. Primary cells (each $n \approx 30$) from five patients with an OSCC and four voluntary healthy donors [primary human keratinocytes (PHK)] showed viscoelastic compliant behavior (Fig. 2*A*) with cancerous cells being >3.5 times more deformable at t = 0.2 second than normal cells ($P < 0.01$). The average compliance values were $D_c(0.2 \text{ second}) = (15.8 \pm 1.5)\ 10^{-3}\ \text{Pa}^{-1}$ and $D_n(0.2 \text{ second}) = (4.43 \pm 0.68)\ 10^{-3}\ \text{Pa}^{-1}$, respectively. The compliance distributions were broadened and had a higher mean value for cancer cells compared with normal cells (Fig. 3*A*). The histologic examination

of the resected cancer tissue of the respective patients clearly confirmed malignancy; the International Union Against Cancer histologic variables (10) are summarized in Table 1. Interestingly, cells from the one tumor without regional lymph node metastases were less compliant than those from tumors with metastases (Fig. 2*B*).

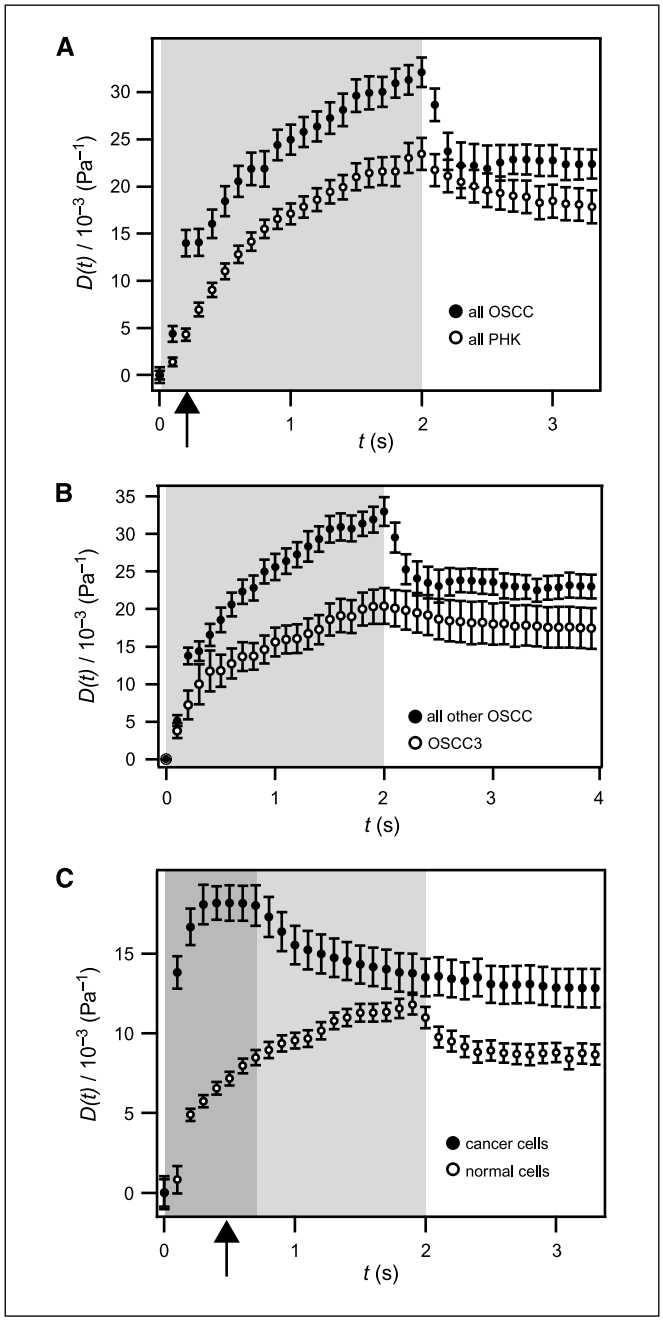

Figure 2. Compliance of normal and cancer cells. *A,* averaged temporal development of the compliance of all primary normal (PHK) and cancer cells (OSCC). Of each sample, n = 10 to 60 cells were measured. *B,* difference in compliance between metastatic and nonmetastatic OSCCs. Averaged temporal development of the compliance of all cells (n = 71) from four tumors that had developed regional lymph node metastases (OSCC1, OSCC2, OSCC4, and OSCC5; Table 1) and the one tumor sample without metastases (OSCC3; n = 13). All five tumors had the same grading. The metastatic cells are more compliant. *C,* averaged temporal development of the compliance of all normal cell lines (OKF-4 and OKF-6) and all cancer cell lines (CAL-33, CAL-27, BHY, and HN). The gray backgrounds indicate the 2.0-s duration of stress application (except 0.8 s for the cancer cell lines in *C*). *Points,* mean; *bars,* SE.

Comparing two normal and four cancer cell lines (Fig. 2*C*), the cancer cells deformed more rapidly on stress application and the extension reached a plateau after ~0.2 second (thus, the stress duration was kept shorter than for the other measurements). The normal cells exhibited a more retarded behavior, where the deformation increased at a slower rate. The mean compliances at t = 0.5 second for all normal and all cancer cell lines were D_n(0.5 second) = (7.18 ± 0.43) 10^{-3} Pa^{-1} and D_c(0.5 second) = (18.2 ± 1.1) 10^{-3} Pa^{-1}, respectively. The cancer cells were >2.5 times more deformable and could clearly be distinguished from the normal cells (P < 0.05). The compliance distributions of the various cell lines with their averages and SDs are shown in Fig. 3*B*, which illustrate the increased average compliance and a larger variance of the cancer cells compared with the normal cells.

Discussion

Assessing malignancy and metastatic risk. The aim of this study was to evaluate the potential for compliance measurements of individual cells to assess oral lesions and to distinguish between normal keratinocytes and squamous cancer cells from the oral cavity. Our results show that both immortalized and primary cancer cells have an increased compliance with a broadened distribution compared with their normal counterparts, which seems independent of the site of origin within the oral cavity.

The increase in deformability is likely caused by a decrease in filamentous actin, which is reduced by 30% in cancerous keratinocytes compared with normal keratinocytes (4), a value that is similar to the difference between normal and cancerous fibroblasts (11). In addition, an altered nucleus to cell ratio and DNA ploidy status (12), leading to a reduced nuclear compliance (13), could in principle contribute to a reduced mechanical resistance of oral cancer cells but seems unlikely given the small strains encountered in the optical stretcher (cf. Fig. 1*D*) as discussed previously (14).

The reduced resistance to deformation of cancer cells compared with normal cells is consistent with previous results where researchers investigated bladder endothelial cells with atomic force microscopy (AFM; ref. 15), breast epithelial cells with μOS (11), and fibroblasts using micropipette aspiration (16), microplate manipulation (17), AFM (18), or μOS (11). This is, to our knowledge, the first validation of this recurring theme with primary cells from a solid tumor.

The benefits of a quantitative, cytopathologic approach to diagnosis have driven the search for molecular-based changes in cancer cells (19). However, no unequivocal molecular marker for early oral cancer detection has yet been identified (2, 19). Analyzing the global cell compliance combines many different possible molecular events, which renders it more robust. This quantitative tactile analysis of cytoskeletal mechanics transfers "clinical palpation" to the cellular level.

The difference in viscoelastic signature between cells from the tumor patients with metastases and the one without (Fig. 2*B*) can be understood by the physical necessity of the metastasizing cells to be motile and, thus, even softer than their nonmetastasizing counterparts. This finding confirms previous results on fibroblast cells (16), breast epithelial adenocarcinoma cells (11), and chondrosarcoma cells (20) measured with micropipette aspiration, μOS, and AFM, respectively. Once corroborated in future clinical

Table 1. Synopsis of patients' clinical palpation status and histopathologic variables according to UICC (10)

OSCC	Age	Palpation status	T class (pT)	Nodal status (pN)	Grade	Type (ICD-O)	Location (ICD-O)
1	71	+	2	1	2	8071-3	C06.0
2	46	+	4	2b	2	8070-3	C02.8
3	45	+	3	0	2	8070-3	C06.8
4	64	+	4	1	2	8070-3	C06.8
5	53	+	2	1	2	8070-3	C04.8

Abbreviation: ICD-O, International Classification of Diseases for Oncology.

trials, cell mechanical measurements might thus provide a means to assess the metastatic potential of primary tumors.

Multiclonality and dedifferentiation. Normal and cancer cells differ not only in their average compliance but also in the width of its distribution. The large variance in compliance values for cancer cells, both primary and cell lines, is distinct from the narrower distributions found for normal cells (Fig. 3). This is consistent with the progression of cancer leading to a multiclonal situation, where the degree of dedifferentiation varies within the tumor. This variance in dedifferentiation is also reflected in cytoskeletal structure and ultimately in the mechanical properties of the cells. A correlation between increasing dedifferentiation and an increase in both cell deformability and its variance has previously been reported for breast epithelial cancer cells (11).

Of note, if we follow this reasoning, the softest cells encountered should be the least differentiated and it might be sensible to search for possible cancer stem cells among the softest cells in a tumor. Because mechanical phenotyping exploits an inherent property of living cells, which remain intact for sorting, this provides the possibility to separate cells of interest (metastatic cells and cancer stem cells) based on their distinct mechanical properties and to

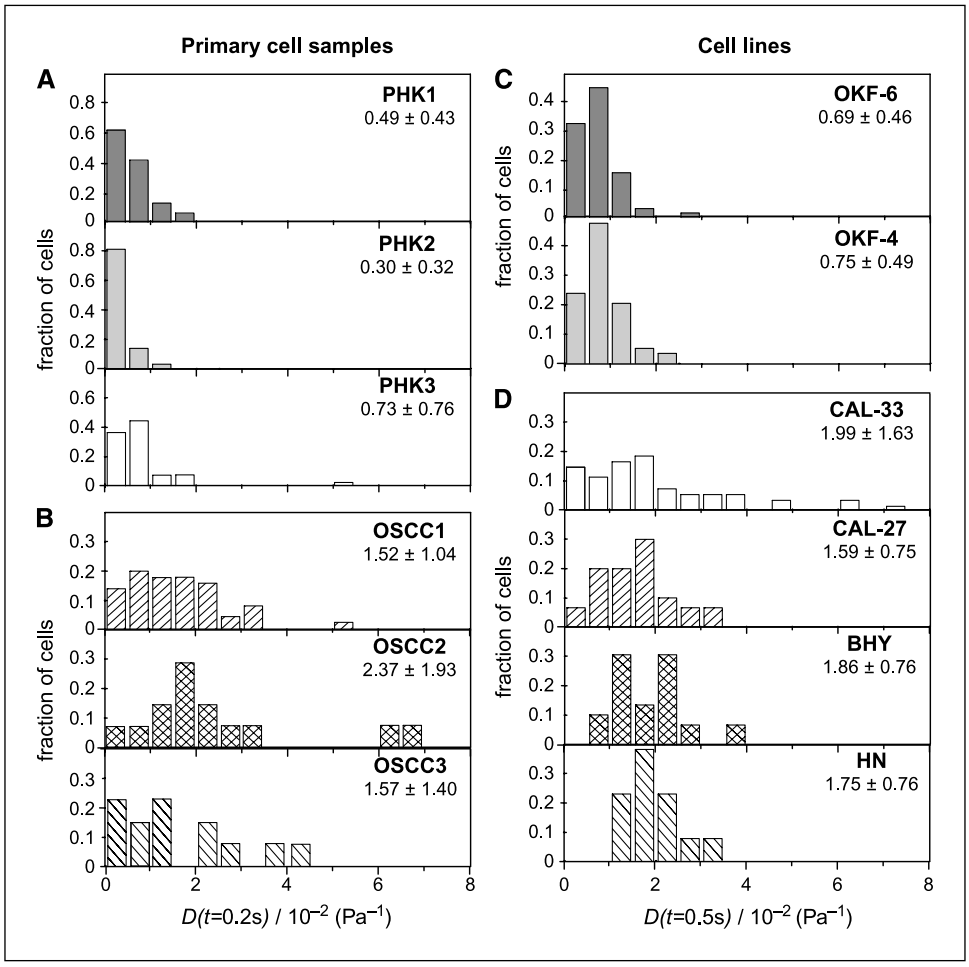

Figure 3. Compliance distributions at particular time points. The compliance distributions at $t = 0.2$ s (*arrow* in Fig. 2*A*) of (*A*) three normal PHK samples and (*B*) three OSCC samples and compliance distributions of (*C*) both normal cell lines (OKF-4 and OKF-6) and (*D*) four cancer cell lines (CAL-33, CAL-27, BHY, and HN) at $t = 0.5$ s (*arrow* in Fig. 2*C*). Normal and cancerous cells are statistically distinguishable in a Mann-Whitney *U* test (primary cells, $P < 0.01$; cell lines, $P < 0.05$). In each panel, the mean and SD of the compliance distribution is given in units of 10^{-2} Pa^{-1}.

establish cell lines for further analysis, identification of molecular markers, or specific drug development.

Possibility for targeted screening. One important aspect of our results is that the assessment of epithelial cells, and maybe even of the metastatic potential of primary tumors, can be inferred from measurements on very small sample sizes (<30 cells). Obtaining cells in a minimally invasive manner (e.g., by brush biopsy) in combination with mechanical phenotyping may enable the development of a simple screening method for oral cancer suitable for broad application. The μOS enables sufficiently high throughput rates and ease of handling so that technicians or dentists could perform these measurements on site. As a consequence, a broad screening of suspicious lesions may lead to earlier diagnosis, which could have significant positive effect on the mortality rate of oral cancer patients.

In summary, mechanical phenotyping using a μOS might be a sensible alternative for quantitative cytologic screening of small samples for an improved and early diagnosis of oral carcinomas and potentially other cancers.

Disclosure of Potential Conflicts of Interest

J. Guck holds a patent on the optical stretcher technique and consults on its potential applications. The other authors declare no competing financial interests.

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