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A general native-state method for determination of proliferation capacity of human normal and tumor tissues *in vitro*

(histoculture/autoradiography/polarization microscopy/image analysis)

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ABSTRACT **An important need in cancer research and treatment is a physiological means *in vitro* by which to assess the proliferation capacity of human tumors and corresponding normal tissue for comparison. We have recently developed a native-state, three-dimensional, gel-supported primary culture system that allows every type of human cancer to grow *in vitro* at more than 90% frequency, with maintenance of tissue architecture, tumor–stromal interaction, and differentiated functions. Here we demonstrate that the native-state culture system allows proliferation indices to be determined for all solid cancer types explanted directly from surgery into long-term culture. Normal tissues also proliferate readily in this system. The degree of resolution of measurement of cell proliferation by histological autoradiography within the cultured tissues is greatly enhanced with the use of epi-illumination polarization microscopy. The histological status of the cultured tissues can be assessed simultaneously with the proliferation status. Carcinomas generally have areas of high epithelial proliferation with quiescent stromal cells. Sarcomas have high proliferation of cells of mesenchymal organ. Normal tissues can also proliferate at high rates. An image analysis system has been developed to automate proliferation determination. The high-resolution physiological means described here to measure the proliferation capacity of tissues will be important in further understanding of the deregulation of cell proliferation in cancer as well as in cancer prognosis and treatment.**

Cancer is a disease involving inappropriate cell division. A realistic model is greatly needed to understand the biology of altered proliferation in cancer as compared to normal tissue and to use information on proliferation capacity as a basis of cancer prognosis and treatment.

Measurements of proliferation capacity of tumors currently are obtained by thymidine-labeling index (TLI), by flow cytometric measurements of cells presumed to be in S phase, or by measuring a nuclear antigen, Ki-67, found in at least certain proliferating cell types (1–11). Whichever method is used, the results obtained show that the higher the S-phase fraction is, the poorer the prognosis. Clinical studies utilizing the TLI procedure have been successful in identifying and determining therapy of a subgroup of lymph-node-negative women with breast cancer having a 48% relapse rate (2). There is therefore great potential value for cancer prognosis, therapy, and biology in determining the proliferative capacity of tumors.

However, as important as the measurement of the TLI seems to be, current methods of measuring the TLI are impractical and are not physiological. For breast tumors, assays must be conducted within approximately 2 hr of surgery, precluding a central laboratory from carrying out the measurement. Generally, the TLI is measured under very high atmospheric pressure in a salt solution to allow penetration of [^{3}H]thymidine into the tissue. Under these conditions the tumor loses viability after a few hours and in many cases it must be assumed that cells capable of cycling are not measured since the time of measurement is so much less than the generation time of the asynchronous cells within the tumor. With regard to other human tumor types, there is very little information regarding measurement of proliferation capacity of surgical specimens.

While flow cytometry provides a more rapid method of measuring cell cycle kinetics and cells can also be assessed for aneuploidy, it presents the following technical problems: (*i*) Dissociation, either mechanically or enzymatically, into a single-cell suspension is required, resulting in loss of ability to observe tissue architecture and the potential selective loss of one or more specific populations of cells. Full evaluation of all the heterogeneous cell types of an individual tumor, including their spatial organization, is of obvious importance in the development of prognostic tests. (*ii*) Flow cytometry does not unambiguously distinguish between S-phase diploid cells and aneuploid resting or nonviable cells. This becomes an important issue, as studies have demonstrated that tumor cell subpopulations that are enriched in aneuploid cells are largely nonviable by dye-exclusion analysis (12–14). (*iii*) In addition, the S-phase fractions of diploid tumors are likely to be underestimated by flow cytometry due to contamination with nonproliferating, nonneoplastic cells. The invasive capacity of diploid cells *in vitro* from primary breast carcinomas has been clearly demonstrated (15).

The nuclear antigen Ki-67 seems to be present in proliferating breast cancer cells (11), but its relevance to other tissue types is not yet known.

Perhaps most importantly, these techniques measure cells in S phase at a single point in time (flow cytometry, Ki-67) or after a very short labeling time (TLI). Thus, these measurements preclude an estimation of the total cell growth fraction of the tumor which may well reflect a more accurate measurement of the proliferative capacity of the tumor.

Importantly, none of the above methods have been applied to systematically measure the proliferation capacity of normal tissues, in particular in comparison with adjacent tumor tissues.

We report here that, with the use of our native-state, three-dimensional, gel-supported primary culture system to measure cell proliferation by autoradiography within the tissues (16, 17) and enhancement of the autoradiography by epi-illuminescence polarization microscopy, growth fraction indices or labeling indices can be determined at high resolu-

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Abbreviation: TLI, thymidine-labeling index.

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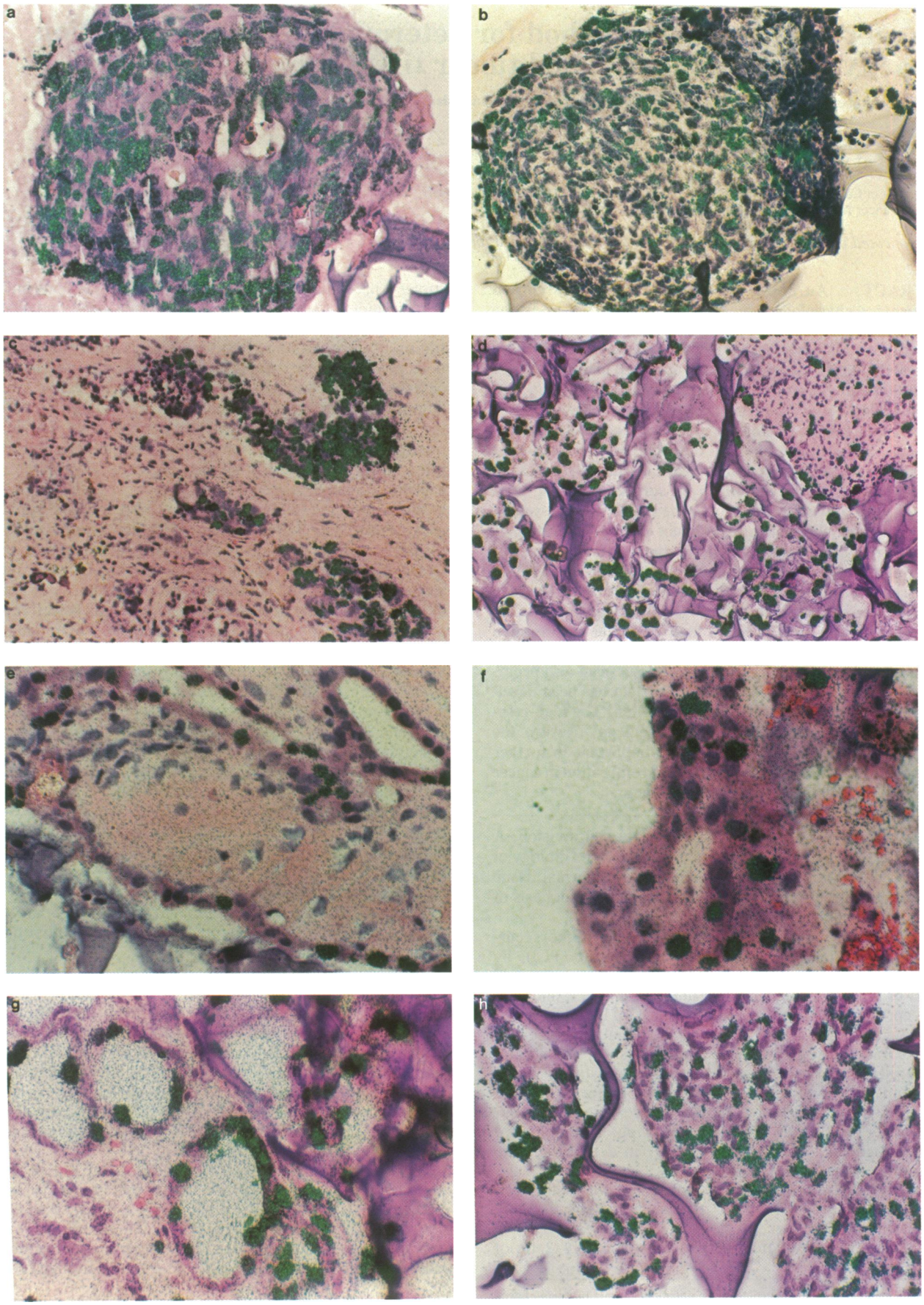

FIG. 1. (*Figure continues on the opposite page.*)

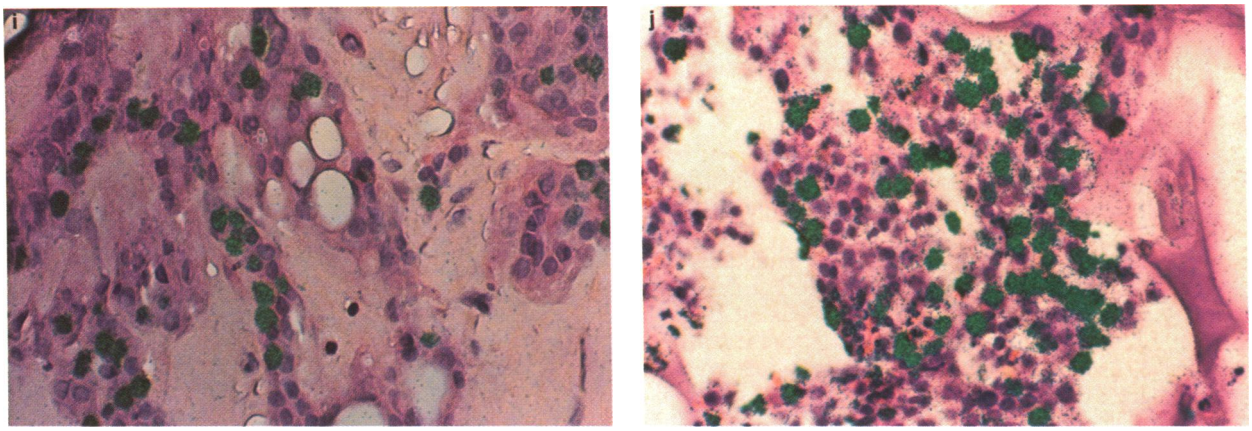

FIG. 1. Cellular proliferation of important tumor types measured in native-state culture in histological autoradiograms analyzed with epi-illumination polarization microscopy. Tumors were in culture for 14 days and were labeled with [^{3}H]thymidine and [^{3}H]deoxyuridine for the last 4 days. Cells with green grains over nuclei are radioactive and therefore proliferating. Original magnification was ×400 for all panels except for *c* and *d*, which were ×200. Final magnifications are ×3300 and ×1700, respectively. (*a*) Colon tumor; (*b*) small-cell lung carcinoma; (*c*) ovarian tumor; (*d*) ovarian tumor; (*e*) pancreas tumor; (*f*) bladder tumor; (*g*) kidney tumor; (*h*) brain tumor; (*i*) parotid tumor; and (*j*) Ewing sarcoma.

tion on all the major types of human cancers with elimination of the problems described above.

Also of major importance, we demonstrate here that our system supports the growth and determination of proliferation of normal tissue.

We also demonstrate in many human tumor specimens, particularly those derived from colon-tumor metastases, ovarian carcinomas, and sarcomas, that the labeling indices in certain areas of the heterogeneous tumors can be extremely high. Normal tissues can also have significant proliferation capacity in our system. Epi-illuminescence polarization microscopy greatly enhances the ability to detect radiolabeled proliferating cells and lends itself to image analysis as shown here.

MATERIALS AND METHODS

Tissues were explanted as described (16, 17). Briefly, after tissues were surgically removed, they were divided into 1- to 2-mm-diameter pieces and placed on top of previously hydrated extracellular-matrix-containing flexible gels derived from pigskin. Eagle's minimal essential medium (MEM) containing Earle's salts, glutamine, 10% fetal calf serum, nonessential amino acids, and the antibiotics garamycin and claforan was added to culture dishes such that the upper part of the gel was not covered.

Cells within the three-dimensional cultures capable of proliferation were labeled by administration of a combination of [^{3}H]thymidine and [^{3}H]deoxyuridine (2 μCi/ml each; 1 Ci = 37 GBq) (17) for 4 days after 10–12 days in culture. Cellular DNA is labeled in any cells undergoing replication within the tissues. After 4 days of labeling, the cultures were washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% Formalin. The cultures were then dehydrated, embedded in paraffin, and sectioned by standard methods.

After the slides were deparaffinized, they were prepared for autoradiography by coating with Kodak NTB-2 emulsion in the dark and exposed for 5 days, after which they were developed. After rinsing, the slides were stained with hematoxylin and eosin.

The slides were then analyzed by determining the percentage of cells undergoing DNA synthesis in treated vs. untreated tumor cultures, using a Nikon or Olympus photomicroscope fitted with an epi-illumination polarization lighting system. Replicating cells were identified by the presence of silver grains, visualized as bright green in the epi-polarization system, over their nuclei due to exposure of the NTB-2 emulsion to radioactive DNA (Figs. 1–4).

For image analysis, a video camera was attached to the microscope. Autoradiograms were viewed under polarizing light without bright-field light, thereby visualizing only the radioactive cells which have exposed silver grains of the nuclear-track emulsion. These cells brightly reflect the polarized light. The image was digitized by a digitizer board and the area of brightness corresponding to the number of labeled or bright cells was calculated as the area of enhanced pixels by the Fas-Com version of the P-See program (The Microworks, Del Mar, CA) run on an IBM PC XT clone. The area of enhanced pixels is proportional to the number of labeled cells.

RESULTS AND DISCUSSION

All human tissues studied here were in culture for 14 days and incubated with [^{3}H]thymidine and [^{3}H]deoxyuridine for days 11–14. In all cases, three-dimensional tissue organization representative of the original tissue is maintained throughout the culture period.

The large majority of tumors cultured in the native-state system demonstrate at least some areas of high cellular proliferation and are intratumorally heterogeneous with regard to proliferation capability. A high degree of detection of radiolabeled proliferating cells is afforded by the epi-illuminescence polarization microscopy, which enhances detection of the audioradiographic exposed silver grains by the scatter of incident polarized light.

Fig. 1*a* illustrates the proliferation capacity of a metastatic colorectal tumor. Note the high labeling in this culture, where more than 90% of the cells in the region shown have proliferated during the labeling period of this relatively undifferentiated colon metastasis to the liver.

Fig. 1*b* demonstrates the proliferation capacity in a lung tumor, in this case one of the small-cell type. Note the maintenance of the two major classes of oat cell types: the classic small cells and the more elongate fusiform cell types. Note also the high degree of cell proliferation.

Fig. 1 *c* and *d* demonstrates proliferation capacity in two ovarian carcinomas. Note the extremely high index of proliferation of the epithelial cells in *c* while the stromal cells are quiescent. Note in *d* the high proliferative capacity of the ovarian carcinoma cells which have invaded the supporting gel matrix. This invasive behavior may mimic the way ovarian tumors frequently invade the peritoneal wall *in vivo*.

Fig. 1 *e–i* demonstrates proliferation capacity in miscellaneous tumors, including those of the pancreas, bladder, kidney, brain, and parotid gland, and a Ewing sarcoma. Note the intricate gland formations containing proliferating cells in many of these cultured tumors.

It is important that distinctions can be made between proliferating epithelial and stromal cell types. For the breast tumor in Fig. 2 *a* and *b*, the epithelial and stromal cells, respectively, are shown to be proliferating.

An additional important observation in these studies is that normal tissues culture and proliferate well. In Fig. 3 tumor and adjacent normal tissue from the breast of patient 431 are compared. Note the extensive cell proliferation present in the normal tissues. Note also the higher level of tissue organization maintained in the normal tissues. With this system it is now possible to compare tumor and normal biology—for example, nutritional requirements, growth factor requirements, and metabolic pathways. Also of critical importance, it is now possible to compare the antitumor selectivity of potential neoplastic agents by comparing tumor and normal response to drugs, using cell proliferation as an end point.

We have demonstrated a generalized system for measurement of proliferation capacity for all the major types of human tissues in relatively long-term culture. As mentioned above, all cultures described in this report have been *in vitro* for 14 days, which is a relatively long period. Greater periods of culture can be achieved with most tissue specimens (data not shown). Greater than 90% of surgical specimens can be cultured and analyzed for proliferative capacity with this system.

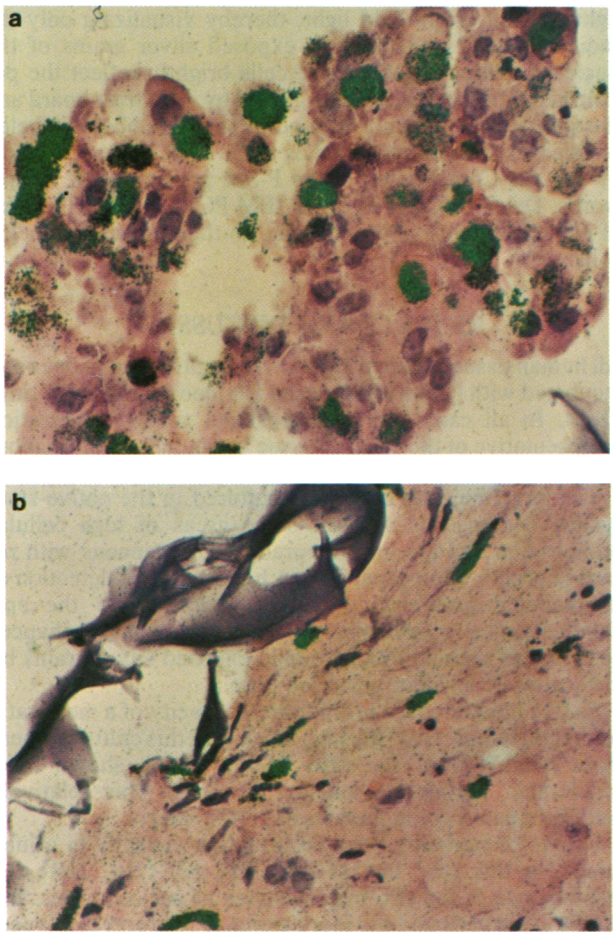

FIG. 2. Autoradiographic determination of proliferation of cancerous epithelial cells and normal stromal cells in tumors in native-state culture. Conditions as described for Fig. 1. Original magnification was ×400; final magnification is ×3600. Proliferating epithelial cells are shown in *a* and proliferating stromal cells in *b*. Note that it is possible to distinguish, with regard to proliferation, between epithelial cells (which in this patient are, by histological criteria, malignant) and proliferating normal-appearing stromal cells.

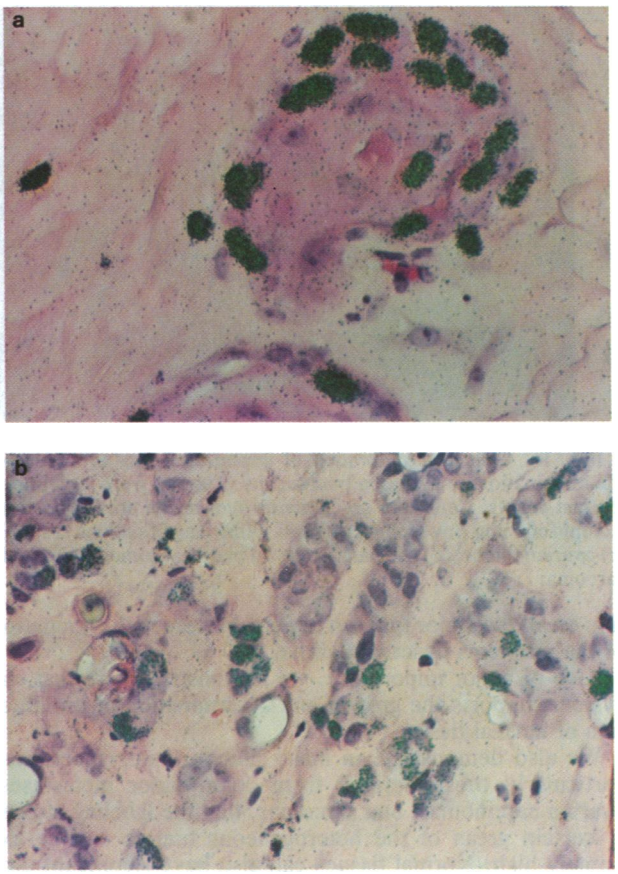

FIG. 3. Cellular proliferation in cancerous (*b*) and normal (*a*) breast tissues *in vitro*. Final magnification, ×3300. Note the relatively high level of proliferation in the normal tissue.

This native-state culture system, with the aid of polarization microscopy, allows a high probability of detecting potential proliferative cells. With the image analysis system described in *Materials and Methods*, the autoradiograms can be automatically analyzed for the number of labeled proliferating cells. With the bright-field and polarized light, the labeled cells of a cultured breast tumor appear bright green (Fig. 4*a*). With polarized light without bright-field, only the labeled cells are visualized (Fig. 4*b*). The image of the labeled cells is then digitized through a video camera and the P-See program (Fig. 4*c*). The area of brightness or enhanced pixels is then automatically determined by the Fas-Com program. The area of enhanced pixels is proportional to the number of labeled cells (data not shown), enabling the automatic counting of labeled, proliferating cells.

An important aspect of the culture system is the use of a flexible extracellular-matrix-containing gel on which to explant the tumors. Other investigators have noted that flexible extracellular-matrix-containing substrata are critical for growth and function of differentiated cells (18–22).

The general principles here are applicable to all types of human tissues, allowing the accumulation of potential important biological and clinically prognostic information. In addition, it should be noted that many of these tumors have high capabilities of cell proliferation. The eventual understanding of the deregulation permissive for such proliferation should be facilitated with the system described here and allow us a deeper understanding of the changes occurring in oncogenesis.

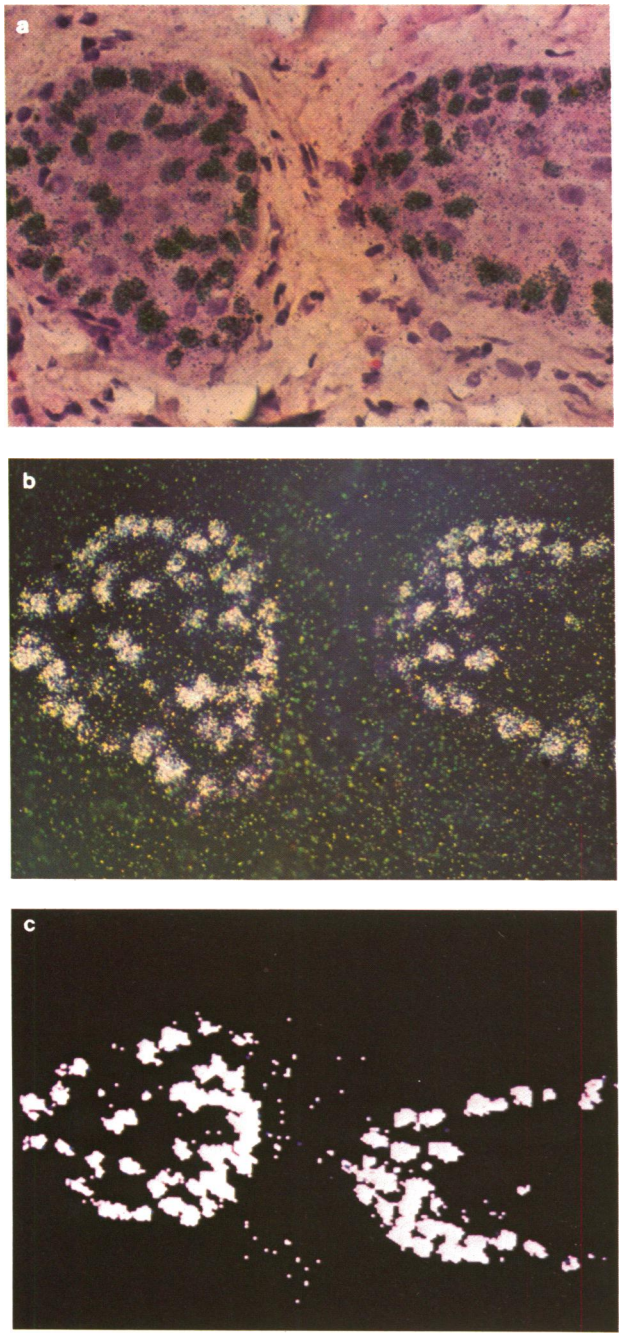

FIG. 4. Computer-assisted automated determination of cell proliferation indices of breast cancer in native-state culture by using autoradiography, bright-field polarization microscopy, and image analysis. Final magnification, ×3300. (*a*) Bright-field and epi-illumination polarization microscopy of autoradiogram. Radioactive nuclei have exposed silver grains, which appear green due to polarization microscopy. (*b*) Epi-illumination polarization microscopy without bright-field light. Only dividing, autoradiographically labeled, cells are visible. (*c*) Digitized processed image of *b* on computer monitor using P-See and Fas-Com programs. Image represents only dividing, autoradiographically labeled cells.

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