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Development of a Stereological Method to Measure Levels of Fluoropyrimidine Metabolizing Enzymes in Tumor Sections Using Laser Scanning Cytometry

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Background: The enzymes thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) influence the activities of fluoropyrimidine anticancer drugs. The sensitivity of cancer cells to capecitabine, which is an oral, tumor-selective pre-prodrug of 5-fluorouracil may correlate better to the TP/DPD ratio than to levels of either enzyme alone. Our goal was to develop a quantitative immunofluorescent method for estimating the levels of TP, DPD, and their ratio in archival tumor sections.
Methods: Mouse anti-TP and rat anti-DPD monoclonal antibodies were used for parallel indirect immunofluorescent staining. The fluorescence was measured using a laser scanning cytometer (LSC; CompuCyte, Cambridge, MA) in single cells and in sections prepared from cell lines and a human tumor. The phantom contouring feature of the LSC provided a stereologic approach for collecting the fluorescence intensity data from sections.
Results: The relative fluorescence intensities measured in single cells or in sections of the cell lines, using single or double labeling, were similar, supporting the suitability of phantom contouring and two-color staining. Sections of the T-24 and ZR-75-1 cell lines placed on the same slide as the tumor section were used as internal standards for fluorescence measurements. The TP/DPD ratios measured in three cell lines correlated well with the cytotoxicity of 5′-deoxy-5-fluorouridine measured in vitro, indicating that the measurements are related to the biological activity of the drug.
Conclusions: Plotting the data as contour maps of the topologic distribution of fluorescence intensities in tumor sections allows subsequent histopathologic examination, which may reveal features of the tumors leading to high or low ratios of these enzymes. In addition, this method can be used for any drug target/metabolic system where the key components are known and suitable antibodies are available. © 2005 Wiley-Liss, Inc.

Key terms: laser scanning cytometry; phantom contouring; quantitative immunofluorescence; stereology

It would be desirable to predict a patient's response to chemotherapy before the start of treatment. In the case of prodrugs, which have to be processed by various enzymes to become active, measurement of the activity/expression of activating enzymes may provide a way to predict the sensitivity of tumor cells to the drug.

Capecitabine (Xeloda) is a prodrug of the widely used antimetabolite 5-fluorouracil (5-FU) and is converted to its active form by a three-step process (1). The final rate-limiting step in its activation is the conversion of 5′-deoxy-5-fluorouridine (5′-DFUR) to 5-FU, which is catalyzed by the enzyme thymidine phosphorylase (TP). This enzyme appears to have higher activity in various tumors compared with adjacent normal tissues (2), which explains the lower systemic toxicity of capecitabine compared with 5-FU and that it is a good candidate as a predictive marker for capecitabine-based chemotherapy. Dihydropyrimidine dehydrogenase (DPD) is the first catabolic enzyme responsible for inactivating 5-FU and expression/activity of this enzyme is inversely correlated with response to 5-FU–based chemotherapy (3,4). Results of a preclinical study have demonstrated that the ratio of TP/DPD activities may correlate better with the sensitivity or resistance of cancer cell xenografts to capecitabine than the levels of TP or DPD alone (5). Similarly, in a recent study, the TP/DPD ratio in patients with primary gastric cancer were determined by the enzyme-linked immu-

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nosorbent assay (ELISA) and showed a significant correlation with 5′-DFUR sensitivity (6).

The most direct approach is biochemical measurement of enzyme activities, but this requires fairly large samples of fresh or freshly frozen tissue and cannot take into account the proportion of normal tissue in a tumor sample. Three indirect assays have been developed to estimate the levels of these proteins in tumor and normal tissues: (a) immunohistochemical assays for examining expression of the enzymes; (b) quantitative polymerase chain reaction (PCR) assays for measuring mRNA levels; and (c) ELISAs also for measuring protein levels. Although enzyme activity measurements, quantitative PCR, and ELISAs provide objective and quantitative results, they do not provide information about the regional or cellular localization of the molecules and they usually require fresh or frozen tissues or cells. The immunohistochemical assay can be done with paraffin-embedded and fixed tumor tissues and provides information about the localization of the antigens, but it is a subjective assay that depends on the judgment of the person reading the slides and is at best only semiquantitative. In this study, our goal was to develop a method that uses a laser scanning cytometer (LSC) and immunofluorescence to combine many of the advantages of the other techniques, and that can provide quantitative measurements of the ratios of these two enzymes in fixed single cells or in sections of paraffin-embedded tumor blocks.

MATERIALS AND METHODS

Cell Preparation and Staining for Single-Cell Measurements

Two established breast cancer cell lines (MDA-MB-231 and ZR-75-1) and one bladder cancer cell line (T-24) in which the activity of TP and DPD is well characterized (5) were chosen as controls. These cell lines represented the extreme low (T-24 and MDA-MB-231) and the extreme high (ZR-75-1) TP/DPD ratios as determined by enzyme activity measurements (5). The cells were harvested from cultures, washed, and suspended in phosphate buffered saline (PBS) at 10^5 cells/ml. One milliliter of this cell suspension was deposited on silanated slides by using a Hettich Universal 16 cytospin centrifuge (Hettich, Tuttlingen, Germany). Slides were air-dried and kept at −20°C until stained.

For primary labeling of enzymes, a mouse anti-human TP monoclonal antibody (1C6-203) and a rat anti-human DPD monoclonal antibody (2H9-1b) were used (both from Roche Diagnostics, Indianapolis, IN, USA). Appropriate species-matched antibodies were used as isotype controls. As secondary antibodies, Alexa Fluor 488 goat anti-rat immunoglobulin G (IgG) (H + L) was used for labeling the rat anti-DPD antibody, and Alexa Fluor 546 goat anti-mouse IgG (H + L) was used for labeling the mouse anti-TP antibody (Molecular Probes, Eugene, OR, USA). For contouring on the cells, TO-PRO-3 (Molecular Probes), a monomeric cyanine nucleic acid stain that is excited by the 633-nm wavelength HeNe laser and its emission detected in the long red channel, was used.

After blocking nonspecific binding sites with Superblock (ScyTek, Logan, UT, USA), slides were incubated at room temperature for 90 min with optimized concentrations of primary antibodies (1:200 for anti-TP and 1:500 for anti-DPD; stock concentration was 1 mg/ml for both). After a washing step in PBS and 30 min of incubation with the secondary antibodies also at room temperature, slides were washed again in PBS and incubated for 15 min with TO-PRO-3 at a concentration of 1 μM. The saturating concentrations for the primary and secondary antibodies were determined with single primary–secondary pairs; for the double-staining protocol, equal volumes of 2× concentrations of antibodies were mixed. Slides were rinsed with distilled water and coverslipped with Prolong antifade solution (Molecular Probes).

Preparation and Staining of Sections of Paraffin-Embedded Cell Pellets and Tissue Sections

Cultured tumor cells were trypsinized and washed three times in PBS, and a tight pellet was prepared by centrifuging the cells at 1,500*g* for 10 min. The cell pellet was incubated in 10% neutral buffered formalin overnight at 4°C. The cell pellets were first embedded in HistoGel (Richard-Allan Scientific, Kalamazoo, MI, USA) and then in paraffin according to standard pathology protocols. The paraffin-embedded cell lines and tissue blocks were cut into 4- to 5-μm-thick sections and one section of each control cell line sample was mounted on the same slide with a tumor tissue section. The tissue used for this study was a human esophageal tumor. Sections were obtained from Asterand, Inc. (Detroit, MI, USA). All tissues provided by Asterand were collected according to standardized protocols. Paraffin-embedded tissues were first fixed in buffered formalin for no longer than 24 h before processing.

Before staining, sections were deparaffinized with xylene, rehydrated with 95% ethanol, placed in preheated DAKO Target Retrieval solution (DAKO Corporation, Carpinteria, CA, USA) and antigen retrieval was performed at 95°C for 25 min by heating in a vegetable steamer.

The staining procedure was the same as that described above for single cells, except that TO-PRO-3 was not used in sections for contouring. This made it possible to use Alexa 647 goat anti-mouse IgG (H + L) (Molecular Probes), which is excited by the HeNe laser, instead of the Alexa 546.

LSC Scanning

Single cells. After staining, slides were analyzed with an LSC (CompuCyte Corp, Cambridge, MA, USA). The LSC was equipped with an Ar ion and a HeNe laser (wavelengths 488 and 633 nm, respectively). The Ar-ion laser line was used for excitation of the Alexa 488 and 546 dyes, and the HeNe was used for the DNA dye TO-PRO-3. For measurements on single cells, contouring was based on the TO-PRO-3 fluorescence detected in the long red channel (>650 nm). Fluorescences of the Alexa 488 and 546 dyes were detected in the green and orange channels, respectively.

Sections. The phantom contouring feature of the LSC (7) was chosen to detect the fluorescence intensity in tissue sections. Phantom contouring is a stereologic, random sampling technique where circular contours are randomly generated by the LSC. The size and density of phantom contours are determined by the operator. They are analyzed by the software in the same way as contoured cells (7). In WinCyte 3.4, which was used for these studies, WinCyte has to be started in the service mode to enable phantom contouring. This is done by double clicking on the login while holding down the "shift" key. In the more recent version, 3.7.1, this feature can be enabled in the setup menu under "Instrument Settings." In our studies, the 10× objective was used, the radius of the phantom contours was set to 10 μm, the minimal distance between the phantom centers to 20 μm (to avoid overlapping of contours), and the number of contours was set to the maximum of 950 per field. The Alexa 488 dye was excited and its fluorescence was detected as described above, and the Alexa 647 was excited by the HeNe laser and its emission was detected in the long red channel.

Data Analysis

The following parameters were used to characterize the enzyme levels. In single cells identified by contouring on fluorescence of the nuclear stain TO-PRO-3, the integral fluorescence related to each enzyme divided by the area of the contour was used to describe the enzyme levels. This corrects for differences in cell size. In sections of cell lines, the integral alone was used because the area of each phantom contour is constant.

Normalization. The measured fluorescence intensities were normalized to those of the ZR-75-1 cell line, which had the highest ratio of TP/DPD enzyme activities of a number of lines studied previously (5).

Contour mapping of TP/DPD ratios in tumor sections. WinCyte 3.4 was used to draw scatter plots of the X and Y positions of the detected cells or phantoms, and regions on the fluorescence integral histograms were defined and assigned different colors, which were displayed on the X–Y plots. However, WinCyte 3.4 graphs can be transferred only using screen capture, resulting in poor quality reproductions for publication purposes. Therefore, files produced by WinCyte 3.4 were exported as ASCII text files. The listmode data were used for creating contour maps by using the "filled contour plot" graph style of Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA) after smoothing the raw data with the built-in three-dimensional data smoother.

MTT Assay for Measuring Cytotoxicity

Sensitivities of the tumor cell lines to 5′-DFUR were determined with the 3-(4,5-dimethylthiazol-2yl)-2,3-diphenyltetrazolium bromide (MTT) assay (8). Briefly, tumor cells were plated into triplicate wells of a 96-well plate at cell concentrations for each line that had been previously determined to be optimal. Two different cell concentrations were plated for each cell line (1,600 and 800 cells/well for ZR-75-1 cells; 800 and 400 cells/well for T-24 cells; and 400 and 200 cells/well for MDA-MB-231 cells). After 24 h of incubation at 37°C in a fully humidified atmosphere of 5% CO_2 in air, increasing concentrations of 5′-DFUR were added. After 72 h of incubation, 50 μl of MTT (3 mg/ml) was added and plates were incubated for 4.5 h before addition of 50 μl of 25% sodium dodecylsulfate at pH 2.0. Plates were incubated overnight, and absorbance was measured at 595 nm using a Tecan Ultra ELISA reader (Tecan Inc., Durham, NC, USA).

RESULTS

Optimization of Labeling and Measurement Conditions

Three cancer cell lines were used to establish conditions for measuring immunoreactive enzyme levels in tumor sections, a bladder cancer cell line (T-24) and two breast cancer cell lines (MDA-MB-231, ZR-75-1). Initial experiments were performed with cells prepared with a cytocentrifuge. The numbers of cells used for preparing the slides were limited so that individual cells were isolated from each other. Nuclei of the cells were contoured by using the DNA dyes propidium iodide or TO-PRO-3. TO-PRO-3 appeared to produce better results in preliminary studies so it was used for all further work. The results of measurements of TP obtained with cells stained only with anti-TP were compared to those obtained with cells stained with anti-TP and anti-DPD (Fig. 1A and 1B). Results are expressed as integral fluorescence of the orange channel divided by the contour area to correct for variability in cell sizes. The relative TP levels in the cell lines measured were similar whether stained with anti-TP alone or with both antibodies, with T-24 having the lowest level of fluorescence and ZR-75-1 having the highest.

During the course of these experiments, it was clear that there was considerable overlap between the green and orange channels that could not be completely compensated for by the WinCyte program. Further, experiments with tumor sections indicated that establishing optimal threshold levels for nuclear contouring was extremely difficult due to the close proximity of the cells and the random orientation of the nuclei in the sections as seen in Figure 2A. There were clusters of multiple nuclei that were contoured and areas where none were contoured. Setting higher thresholds for the contours led to diminishing numbers of cells that were detected. Therefore, the phantom contouring capability of the LSC was used to provide unbiased random sampling of areas in the sections so that all areas had an equal probability of being sampled (Fig. 2B). The results of the double-stained sections using the same fluorochromes as used in the experiment described above are shown in Figure 1C. Because the area of the phantom contour is constant, the results are expressed as integral fluorescence. The histograms are narrower than those shown in Figure 1B, appearing similar to those shown for the single labeled, isolated cells (Fig. 1A).

Because TO-PRO-3 was no longer needed for contouring the nuclei with stereologic analysis, the far red chan-

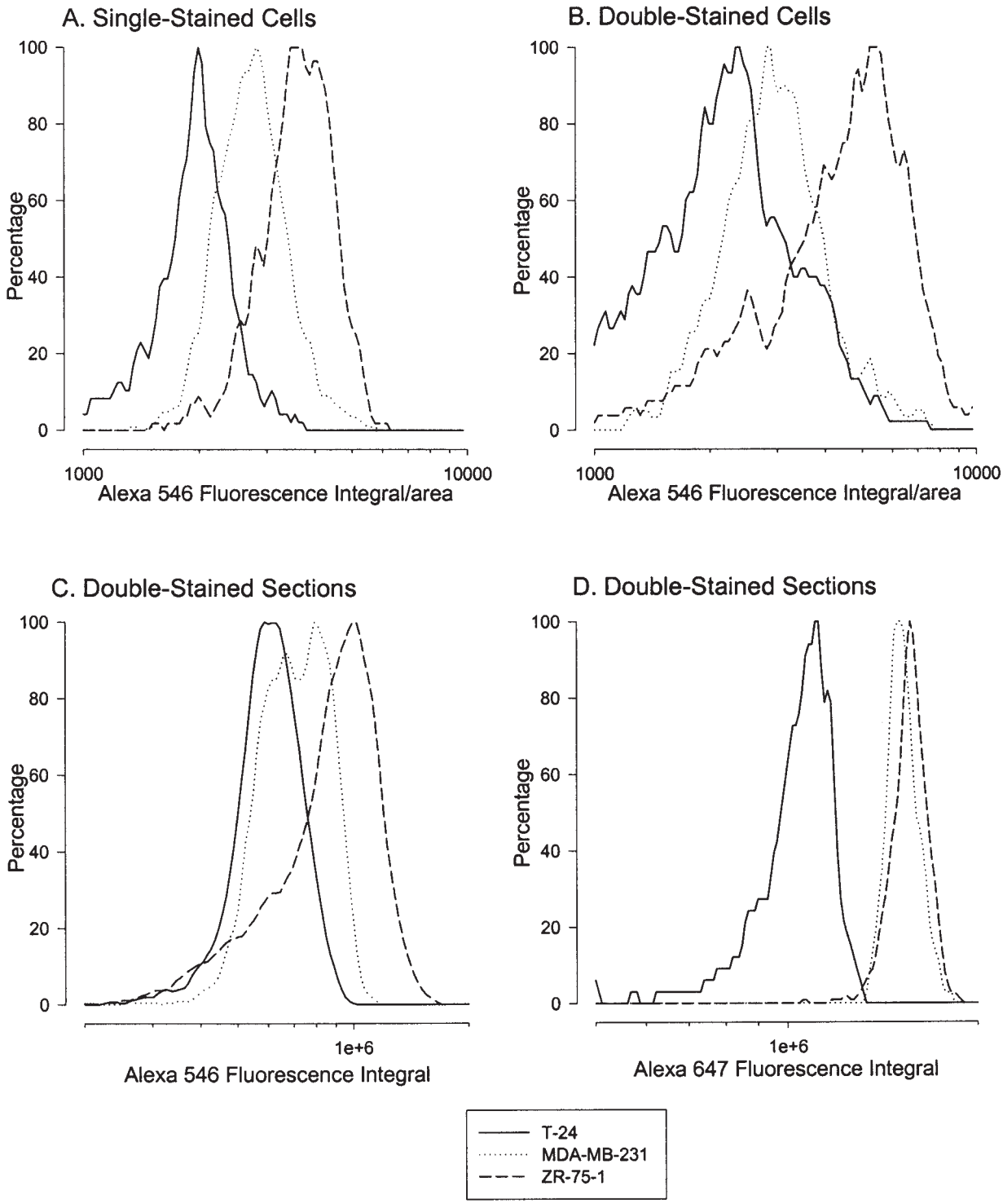

FIG. 1. Comparison of staining for TP in single cells and sections using different fluorochromes. **A:** Single cells stained with Alexa 546–labeled secondary antibody to detect binding of anti-TP. **B:** Single cells double stained with Alexa 546–labeled secondary antibody for detection of anti-TP and Alexa 488–labeled secondary antibody to detect binding of anti-DPD (Fig. 3A). **C:** Sections double stained with Alexa 546–labeled secondary antibody for detection of anti-TP and Alexa 488–labeled secondary antibody to detect binding of anti-DPD. **D:** Sections double-stained with Alexa 647–labeled secondary antibody for detection of anti-TP and Alexa 488–labeled secondary antibody to detect binding of anti-DPD (Fig. 3B). Solid lines represent results obtained with T-24 cells, dotted line represents results with MDA-MB-231 cells, and dashed lines represent results with ZR-75-1 cells.

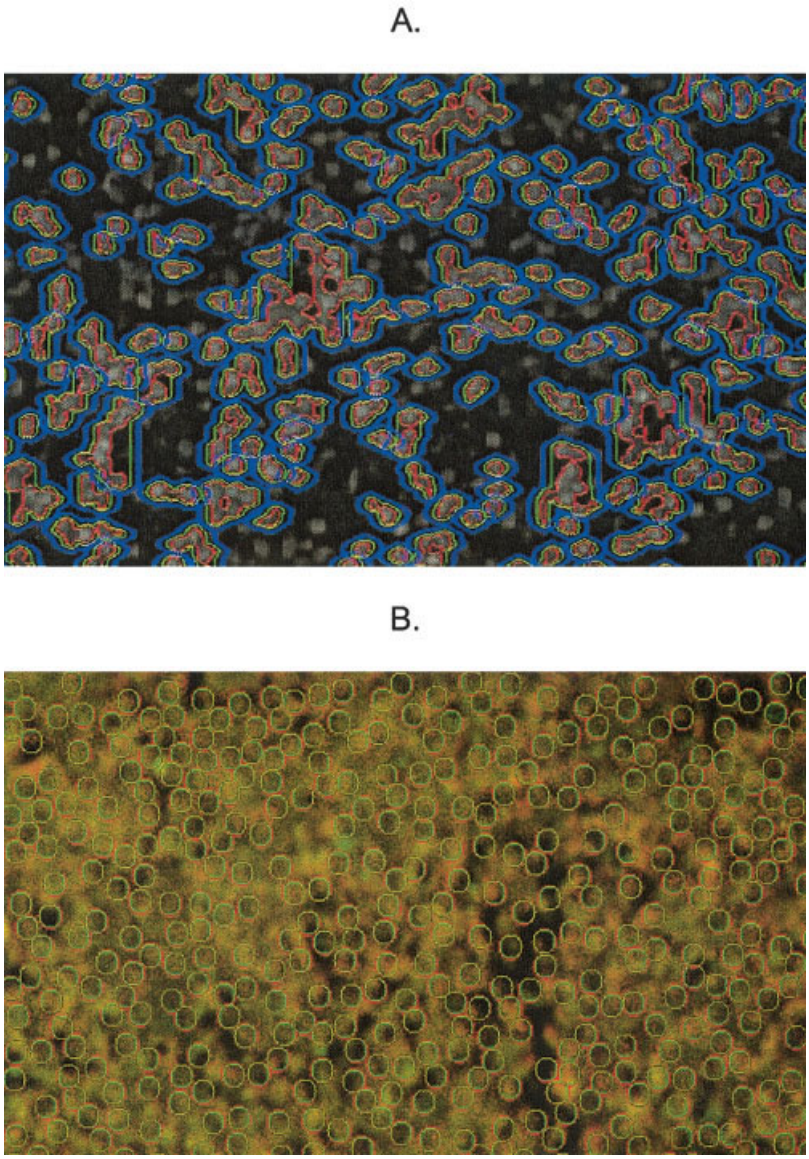

FIG. 2. Scan data display images of standard contouring (**A**) and phantom contouring (**B**) on sections of an esophageal tumor.

nel was used for detecting TP as shown in Figure 1D, where Alexa 647 instead of Alexa 546 was used as the secondary label for TP in cell line sections. The Alexa 488 secondary label remained the same for staining DPD. The use of the two lasers for these measurements, which scan sequentially, means that there is no overlap between the two labels.

The DPD measurements in single cells stained with only anti-DPD (Alexa 488) and in sections stained with TP and DPD antibodies indicated that both protocols produced similar results with respect to the relative enzyme levels in the three lines (Fig. 3A and 3B). We measured the lowest DPD level in the T-24 cells and the highest in the MDA-MB-231 cells.

The ratios of TP fluorescence integral (as measured with Alexa 546 or Alexa 647) to the DPD fluorescence integral (Alexa 488) were calculated directly by WinCyte. Representative histograms are shown in Figures 4A and 4B for the ratio measurements in single cells with Alexa 546/488 and in cell line sections with Alexa 647/488. The highest TP/DPD ratio was seen in ZR-75-1 cells, whereas T-24 and MDA-MB-231 cells showed a much lower ratio of TP/DPD levels.

Based on the results of several studies with these three cell lines, differences in fluorescence intensities for the T-24 and ZR-75-1 cell lines were reproducibly distinct and well separated. Thus, these cells appear to be useful standards for comparing the levels of TP and DPD in tumor sections.

Correlation Between 5′-DFUR Sensitivity and Immunofluorescence Measurements

To demonstrate that the immunofluorescence measurement of the enzyme levels is related to biological activity,

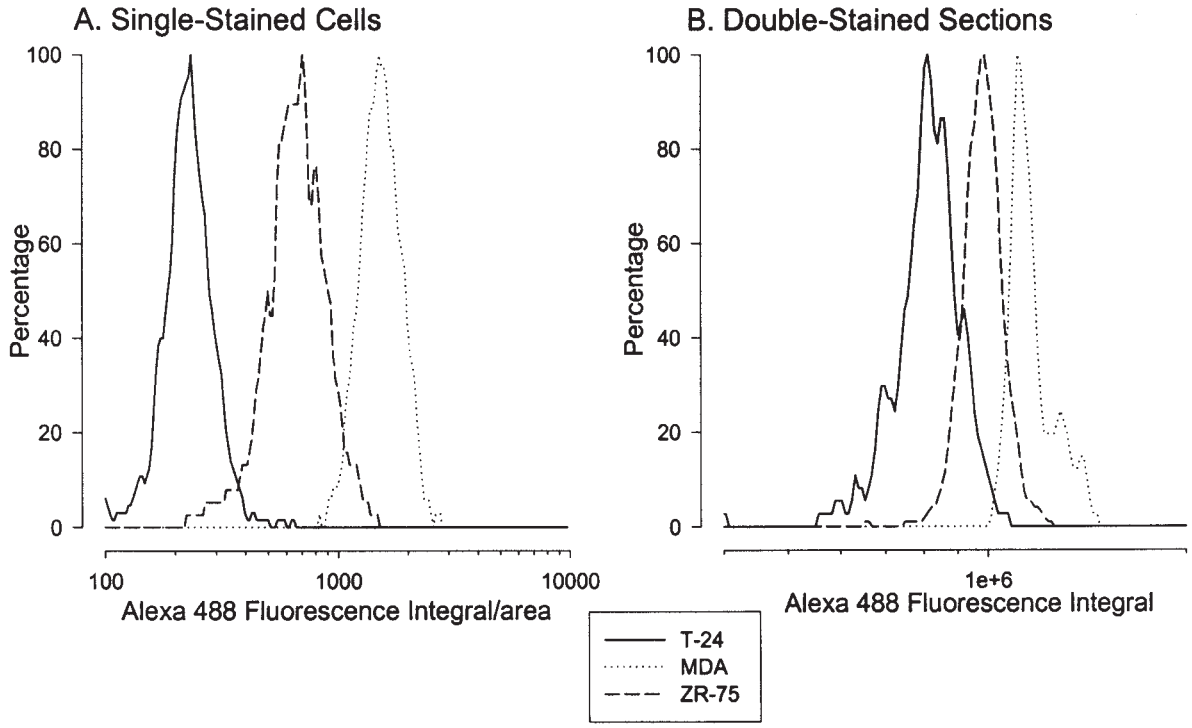

FIG. 3. Comparison of single staining in single cells and double staining in sections for detection of DPD. **A:** Single cells were stained with Alexa 488–labeled secondary antibody to detect binding of anti-DPD (Fig. 1A). **B:** Sections were double stained with Alexa 647–labeled secondary antibody detection of anti-TP and Alexa 488–labeled secondary antibody to detect binding of anti-DPD (Fig. 1D). Solid lines represent results obtained with T-24 cells, dotted line represent results with MDA-MB-231 cells, and dashed lines represent results with ZR-75-1 cells.

results were compared with 5′-DFUR cytotoxicity measurements determined with the MTT assay. ZR-75-1 cells were the most sensitive to 5′-DFUR (50% inhibitory concentration [IC_{50}] = 38 μM), whereas T-24 cells were the least sensitive (IC_{50} = 200 μM). The MDA-MB-231 cell line showed an intermediate level of sensitivity (IC_{50} = 120 μM). There was good correlation between the TP/DPD ratios measured with the LSC (normalized to the ZR-75-1 cell line) and the sensitivities of the cell lines (log IC_{50}) as depicted in Figure 5.

Estimation of Enzyme Levels in a Tumor Section

Figure 6 shows the analysis of the TP/DPD ratio in a tumor section mounted on the same slide as sections of the three standard tumor cell lines. Scan areas were selected for each section and scanned with the Ar-ion and the HeNe lasers (Fig. 6A). The scanned areas were not set exactly to the size of the sections, so the green fluorescence was used for gating out areas where there was no tissue (Fig. 6B and 6C). Histograms of the ratio of long red to green fluorescence (ratio of TP/DPD) show that the TP/DPD ratio in the ZR-75-1 cell line is higher than in the other two cell lines, as expected, and that the heterogeneity of the TP/DPD ratio in the tumor sample is much higher than in the sections of the cell lines (Fig. 6D), as expected.

To examine the topologic distribution of TP/DPD ratios in the tumor sample, different colors were assigned to different ranges of TP/DPD ratios on the histogram with the WinCyte software (Fig. 6E). A scattergram of X position versus Y position was made to show the topologic expression of the TP/DPD ratios (Figs. 6F and 7). To improve the quality of this map, we used the SigmaPlot program, as described in Materials and Methods, to make a contour map of the tumor section (Fig. 6G). The scattergram produced by WinCyte and the contour map made by SigmaPlot show similar distributions of the high and low ratios in the section.

DISCUSSION

We have developed a method for measuring the ratios of immunoreactive proteins directly in sections of formalin-fixed, paraffin-embedded tissue using the LSC. We concentrated on the enzymes TP and DPD, which are responsible for activating the prodrug capecitabine to its active form, 5-FU, and for inactivating 5-FU because there is evidence from preclinical (5) and clinical (6) studies suggesting the ratio TP/DPD correlates better with sensitivity to capecitabine and 5′-DFUR than with levels of either enzyme alone. None of the currently available assays such as enzyme activity measurements, immunohistochemical analysis, quantitative PCR, or ELISA are able to provide quantitative estimations of this ratio in sections from formalin-fixed, paraffin-embedded tumor blocks, which are routinely used for clinical diagnoses.

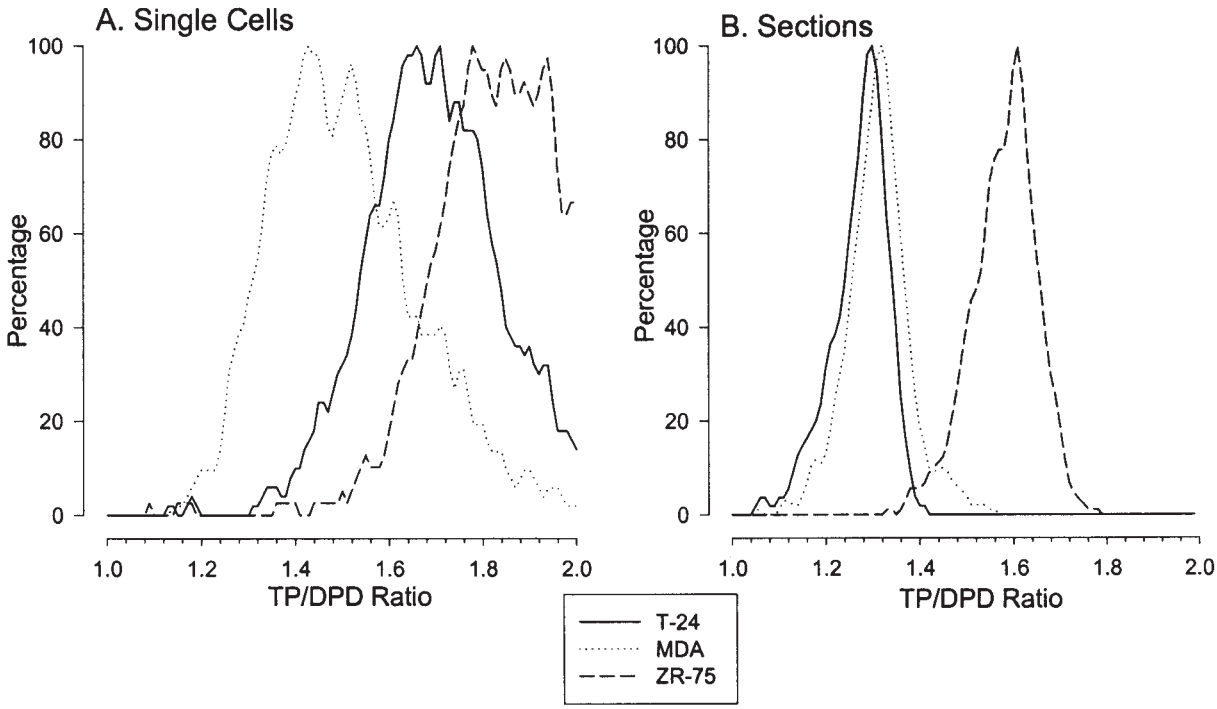

FIG. 4. Comparison of TP/DPD ratios in single cells and in sections. **A:** Single cells were stained with Alexa 488–labeled secondary antibody for detection of anti-DPD and Alexa 546–labeled secondary for detection of anti-TP. **B:** Sections were stained with Alexa 488–labeled secondary antibody for detection of anti-DPD and Alexa 647–labeled secondary for detection of anti-TP. Solid lines represent results obtained with T-24 cells, dotted line represent results with MDA-MB-231 cells, and dashed lines represent results with ZR-75-1 cells.

The advantages of the LSC for this assay is that it is a microscope-based system and therefore ideal for use with tissue sections. Further, this instrument stores the X–Y location of each measured event and has the capability of relocating these events, so that slides can be restained for morphologic examination and the areas of interest readily found (fig. 7). In the past, detection of cells on slides was usually performed by contouring for the whole cell on the basis of forward light scatter or for the cell nucleus on the basis of a fluorescent DNA dye. This contouring works best when individual cells are spatially well separated (e.g., cytospin preps or cytology specimens), although it has been used in some studies on tissue sections (9–11). For contouring epithelial tumor cells, use of a fluorescent DNA dye seems to be most suitable (12). We used TO-PRO-3 in our initial studies with the cell lines because it is excited by the HeNe laser and its emission is detected in the long red channel. Thus, it was possible to simultaneously measure the fluorescence intensity of the Alexa 488 and 546, which are excited by the Ar-ion laser and are detected in the green and orange channels, respectively. However, because the excitation peak of Alexa 546 is far from the 488-nm wavelength of the Ar-ion laser, the absolute fluorescence intensity of Alexa 546 was relatively dim, resulting in a decreased signal-to-noise ratio and decreased accuracy.

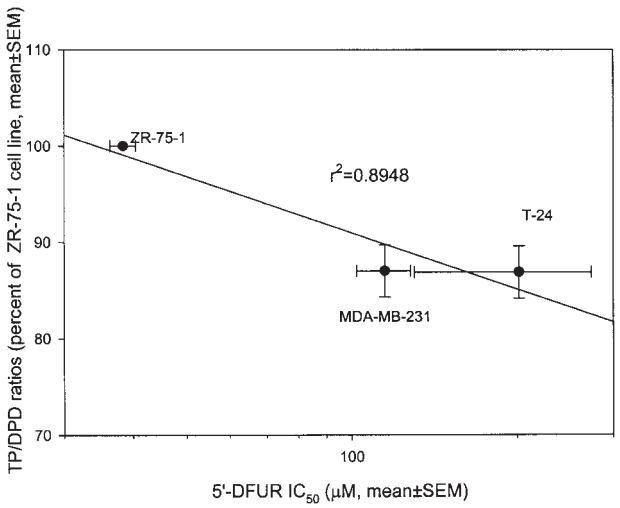

FIG. 5. Correlation between the TP/DPD ratios measured with the IF-LSC method in single cells and the 5′-DFUR sensitivity of ZR-75-1, MDA-MB-231, and T-24 cell lines measured by the MTT assay. The median of the ratio of the integral fluorescence measured for TP and DPD was normalized to the ZR-75-1 cells. IC_{50} and normalized fluorescence intensity values are the averages of at least three separate experiments. SEM, standard error of the mean.

We found it very difficult to contour cells in most tumor sections based on nuclear staining. This is because contouring on the nucleus and setting a fixed distance for the peripheral contour so that at least most of the cytoplasm of each cell could be included does not work well with

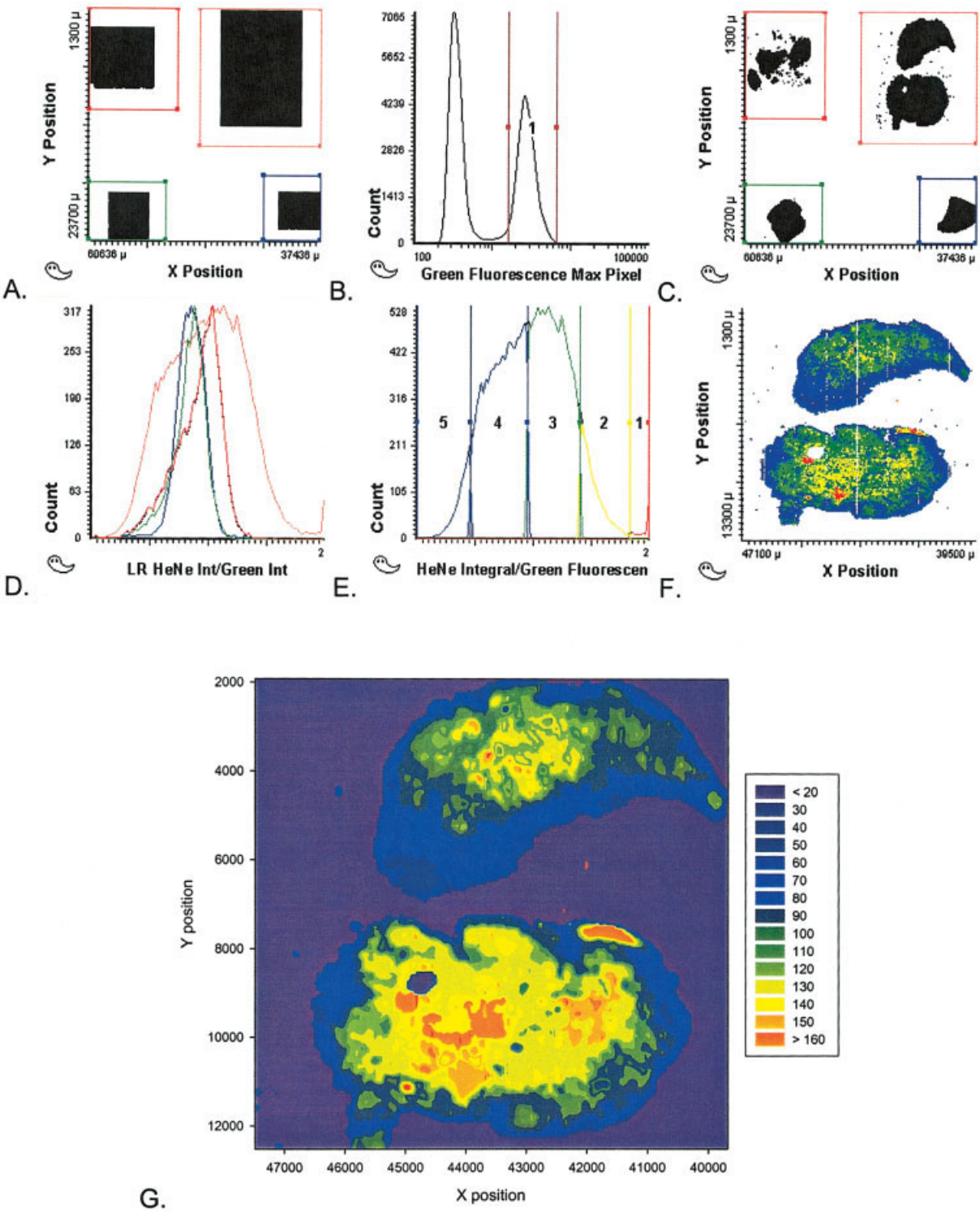

FIG. 6. Contour mapping of the ratio of fluorescence intensities in a tissue section. **A:** Distribution of all phantom contours showing the actually scanned areas. Three cell line sections and a tissue section were mounted on the same slide (ZR-75-1, upper left; MDA-MB-231, lower left; T-24, lower right) and tissue section (upper right). **B:** Separating the non–tissue-containing areas from the tissue-containing parts based on the higher green fluorescence maximum pixel values of the tissue-containing areas. **C:** Distribution of gated phantom contours shows the shape of the tissue pieces mounted on the slides. **D:** Histograms of the ratio of fluorescence integrals characterizing TP and DPD. Colors of curves correspond to the colors of the regions on the previous scattergrams. **E:** Cells in five regions on the histogram were colored with different colors. **F:** X–Y scattergram produced by WinCyte showing the two-dimensional distribution of the colored cells **G:** Contour map of the tissue section as made by SigmaPlot (Material and Methods). Scaling is in percentage of the median of control ZR-75-1 cells.

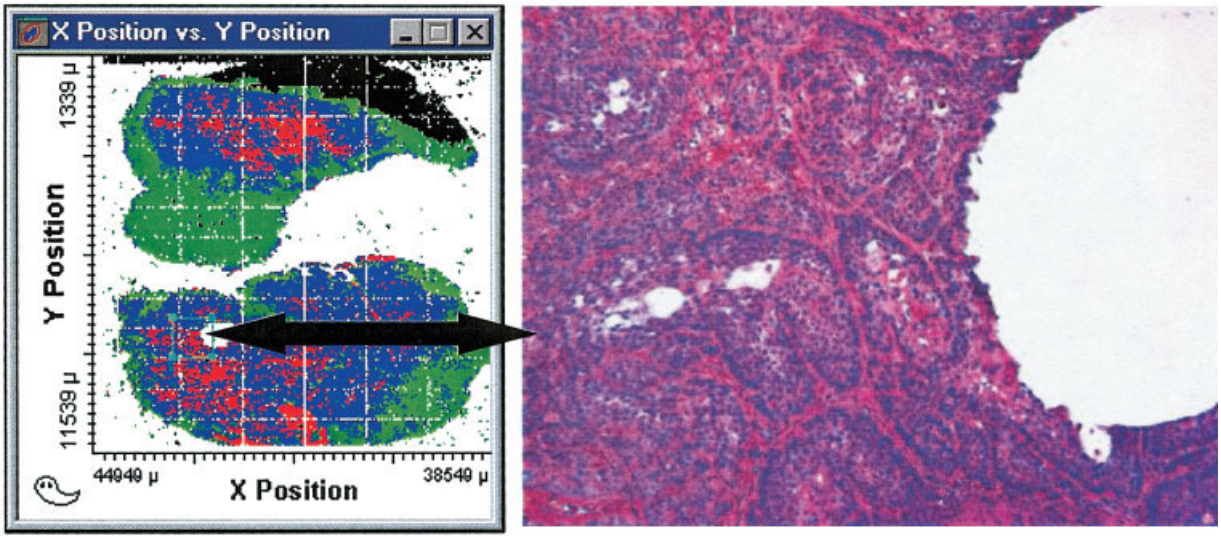

FIG. 7. Relocation of area of interest. Contour map on the left (Fig. 6F) with arrow locating area depicted in the photomicrograph shown in the right panel.

the non-uniform distribution of nuclei in the cells and the general heterogeneity of cells in the sections. In our experience with the tumor cell lines on cytocentrifuge preparations, increasing the cell density to greater than 3×10^5 cells/cm^2 made the segmentation of single cells difficult. Recently, Gerstner et al. (13) described a technique for the LSC, named "multiple thresholding." This method uses conventional propidium iodide contouring on cells of frozen human lymphatic tissue sections with scanning the same areas at five to eight different threshold levels and merging the data. However, such procedures work well for lymphatic tissues and other tissues in which the cells have relatively uniform size and spacing, but not for most cancer tissues in which the cells are very heterogeneous.

The phantom contouring feature of the LSC provides a stereologic approach to measure features of the sections. Stereology is a group of techniques for obtaining quantitative estimates of various parameters from tissue sections and is widely applied in neuromorphology. The basis of stereology is statistical sampling of the sections, and phantom contouring represents an ideal design-based, unbiased sampling protocol (14). Phantom contours are placed randomly on the scanned area in user-defined number, size, and density. The fluorescence data collected from each phantom contour are stored in a listmode file together with the positional information in the same way as cells contoured in the conventional manner. Using these data, it is possible to characterize the fluorescence of different areas on sections. This approach can provide much more accurate estimates of fluorescence such as those obtained using method-based sampling, an example of which is standard contouring on nuclei, because it is based on assumptions about the regularity of cells that may not be true for all areas of the section being examined and therefore is a biased sampling technique (14).

Further, use of phantom contouring allowed us to switch from Alexa 546 to Alexa 647 for one of our secondary antibodies so that we could use separate lasers to excite each of our markers. The sequential measurement of the two fluorochromes resulted in an improved signal-to-noise ratio without the need for compensation.

The order of TP and DPD levels and the TP/DPD ratios measured in the cell lines using the different staining and contouring methods were comparable with the exception of the MDA-MB-231 line. One factor that might influence differences between the two measurements is the use of Alexa 488 labeling, which is measured with the green filter; thus autofluorescence of the cells and tissues could influence the results. Our results are in good agreement with the enzyme activities and ratios measured in these cell lines previously (2). The LSC measurements showed good correlation with the in vitro sensitivity of the lines to 5′-DFUR. This functional correlation appears to validate the use of an immunoreactive protein as an indirect measurement for activity of these enzymes.

The absolute values of the measured immunofluorescence are dependent not only on the levels of the target proteins in the tissue sections but also on the staining conditions and instrument settings of the LSC. For the method to be useful with clinical specimens, it is important to have internal standards that will allow comparison between assays of different tissue samples. The use of sections of well-characterized cell lines mounted on the same slide with the investigational tissue sample provides such internal standards and improves the reliability of the measurements.

The quantitative measurement of TP, DPD, and their ratio in fixed tumor tissues makes it possible to perform retrospective studies to define selection criteria for patients who most probably will respond to capecitabine therapy. The advantage of this method for clinical studies

is that it can be performed with materials that are taken and processed for diagnostic purposes. Other quantitative methods for measuring levels of these enzymes, such as PCR and ELISA, require that biopsy materials be snap frozen in liquid nitrogen or assayed immediately, whereas general clinical practice is to fix materials with formalin and embed in paraffin so that the histology is preserved. The LSC can be used with needle biopsy samples because very few cells are required for the analysis as has been demonstrated with the elegant immunophenotyping method of Clatch and Foreman (15) for needle biopsy specimens of lymphomas.

Phantom contouring, although it provides more accurate measurement of fluorescence over the entire section, no longer provides direct cellular measurements as one can obtain with dispersed cells on cytocentrifuge slides. However, it is still possible to identify regions of interest such as those with the highest or lowest fluorescence ratios and relocate these areas after staining for morphologic assessment of the cellular composition. Thus, questions such as whether the enzymes are more highly expressed in tumor areas, stromal areas, or areas with infiltrating inflammatory cells can be addressed. In addition, this method is not restricted to the fluoropyrimidine pathway but can be used for any drug target/metabolic system where the key components are known and for which suitable antibodies are available.

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