

# Image Analysis of Extracellular Matrix Topography of Colon Cancer Cells

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## BIOGRAPHY

Dr Sarah Glover received her medical degree from Midwestern University in 1998 and completed training in internal medicine and gastroenterology at the University of Illinois at Chicago. Her current research focus is on the role of the extracellular matrix topography in post neoplastic transformation and metastasis in colon cancer.



## ABSTRACT

Scanning electron microscope stereoscopy (SEM-S) has been used for many years to characterize 3D features of non-biological and biological specimens. Traditionally, data production by SEM-S required tedious manual measurements. Digital image processing has led to the development of software for mathematically characterizing 3D surfaces. These programs greatly simplify SEM-S and provide details about features of a specimen including its area, volume, and roughness. We report the use of SEM-S to illustrate and quantify the topographical changes that occur in the extracellular matrix (ECM) as a colon cancer de-differentiates and thus provide new insight into how ECM topography regulates colon cancer.

## KEYWORDS

light microscopy, histology, scanning electron microscopy, stereoscopy, image analysis, colon cancer, extracellular matrix, roughness

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## INTRODUCTION

Since the advent of scanning electron microscopy (SEM) in the 1930s, scientists have had the ability to gain a three-dimensional perspective of both non-biological and biological specimens. However, images obtained with SEM represent 3D images projected onto a 2D plane. Thus, depth information is lost. In the early 1970s, a method was devised which utilized the parallax-shift equation to obtain information about depth from SEM images obtained at the horizontal and several degrees from the horizontal. While this method provided depth information, it also required time consuming measurements by hand followed by laborious calculations [1].

Recently, commercial software has become available to alleviate this problem by taking advantage of advances in digital imaging technology. Photomicrographs are obtained at the horizontal and at several degrees from the horizontal. These images are then imported into a software program that automatically detects corresponding feature points in the different images and derives 3D information from the disparity of the features. The result of this reconstruction is a digital elevation model (DEM) that provides a height value for each pixel in the input images. Using this DEM, metrically correct three-dimensional models of a specimen are reconstructed allowing for numerical information to be obtained. This information includes the roughness profile, areas and volumes of the acquired surface. Roughness measurements are calculated according to ISO standards.

In this article, automated SEM-S technology was used to characterize the extracellular matrix (ECM) underlying colon cancer cells at various stages of de-differentiation. In cancer,

de-differentiation has been linked with a decreased likelihood of survival and a greater chance that the cancer will spread to other organs [2,3]. Using SEM-S software, the topographical features of colon cancer ECM were quantified with the aim of identifying features that might serve as novel physical or mechanical factors regulating colon cancer de-differentiation. As shown in the Results section, profound differences in topography were observed as a cancer becomes less like the normal tissue whence it came. These findings have implications not only for improving our understanding how colon cancer grows and metastasizes but also for bettering our understanding of the role of the ECM topography in cancer and development in general.

## MATERIALS AND METHODS

### Tissue specimens

Human colon cancers were obtained through the NCI Cooperative Human Tissue Network (CHTN). Prior to obtaining specimens through this network, an IRB was approved by the University of Illinois Institutional Review Board. This IRB is exempt because no patient identifying characteristics are known or included as a part of these studies. Specimens were received from the CHTN in OTC media and stored at -80°C until use.

### Scanning electron microscopy

The extracellular matrix underlying both malignant and non-malignant colonic epithelia was prepared for scanning electron microscopy as previously described [4]. Frozen tissue samples were thawed by immersing in sterile phosphate buffered saline (PBS) at 37°C for 30 min, followed by incubating in Hank's balanced salt solution (HBSS) for 10 min. The

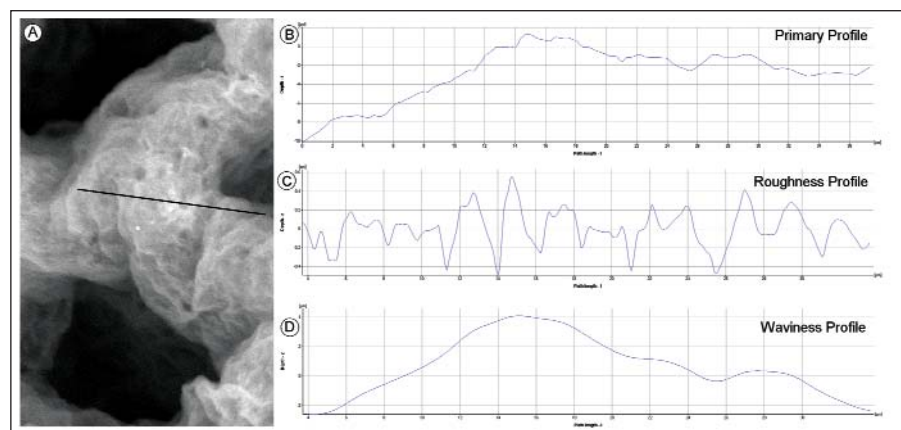


Figure 1:

Difference between roughness and waviness. (A) SEM image of ECM underlying normal colonic epithelia. HPW = 50  $\mu$ m. The line shows the profile sampled. (B) Primary curve. (C) Roughness curve. (D) Waviness curve. Data in (B-D) were generated using MeX SEM-Stereoscopy software.

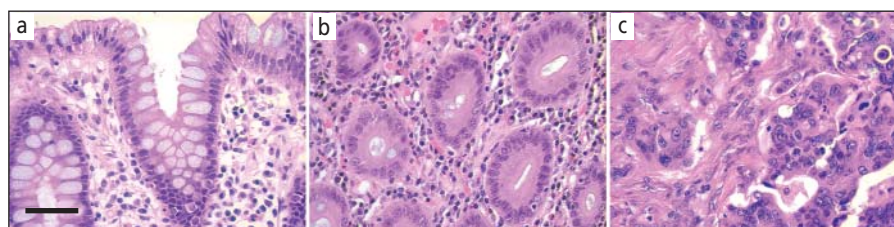
epithelial surface was gently stroked with a 13-mm glass coverslip. The tissue was then incubated in 10 mM dithiothreitol in PBS at RT for 30 min. This was followed by a second incubation with HBSS followed by gentle scraping with a glass coverslip. The tissue was then subjected to two rounds of incubation with 10 mM EDTA in PBS for 30 min at 37°C followed by HBSS for 30 min. The tissue was finally fixed with Trump's fixative (80 mM sodium monobasic phosphate, 67.5 mM sodium hydroxide, 3.75% formaldehyde, and 1% glutaraldehyde in water) for at least 1 hour. Samples were then washed with distilled water and prepared for SEM by alcohol dehydration. Samples were finally dried using hexamethyldisilazane (HMDS). Uncoated specimens were glued onto metal stubs with carbon-coated tabs. SEM images were obtained using a Hitachi S-3000N scanning electron microscope at 20 kV in variable pressure mode at 10 Pa.

### Surface reconstruction

To three-dimensionally reconstruct the ECM topography, stereopair images were obtained at 0 and +7° from the horizontal and input into the MeX image analysis software program (Alicona Imaging, Graz, Austria) [5]. The tilt angle along with the working distance and the size of image pixels were also input for proper calibration. Reconstruction was then performed in two steps. First, corresponding points were extracted from the stereoscopic images. Secondly, metrically correct 3D points were calculated using the geometric relationships from the SEM identified in the first step. While the data presented in this paper were generated from stereopairs, MeX has an extended method called the TriCreator that utilizes three input images with different tilt angles. This method provided even more accurate height measurements [6] since it permitted estimation of true tilt angles.

### Roughness analysis

There are many different ways to characterize surfaces and to compare them to each other. Visual comparison represents the most common way. In addition, various roughness parameters that have been previously identified as important to regulating cell motility, adhesion, and morphology were quantified for each tissue type [7-11]. These roughness parameters were directly calculated using the MeX



**Figure 2:** Histological appearance of colonic mucosa. (a) Well-differentiated colon cancer. (b) Poorly differentiated colon cancer. (c) Normal colonic mucosa is characterized by confinement of cells to well-defined crypts. In (a) crypt architecture is maintained for the most part but there is an obvious change in the cytonuclear ratio of the cells compared with normal colonocytes. Poorly differentiated colon cancer cells are very loosely organized and have large open-faced nuclei. Scale bar = 10  $\mu$ m.

software and were derived from surface profiles that are extracted from the reconstructed 3D models.

The extraction of roughness parameters from surface profiles [12-13] is based on the decomposition of a primary profile or primary curve into a roughness profile that contains the high frequency information and a waviness profile that contains the low frequency information (Figure 1). To understand the meaning behind each of these, it is helpful to think of a highway. The highway is a surface with filter components corresponding to hills, valleys or speed bumps. The speed bumps correspond to finer irregularities of the surface texture or roughness. The hills and valleys represent irregularities that are more spaced out and correspond to waviness. Finally, the hills, valleys and speed bumps together make up the primary curve [14].

Since the results in this study were derived from the roughness curve, picking the Lc value was of utmost importance. The Lc value is the specification of the wavelength that separates the roughness profile from the waviness profile [12]. The Lc value for the results presented in this paper was four  $\mu$ m and the length of the roughness profile was 300  $\mu$ m. In addition to the Lc value, the software also included a filter to ensure the calculations were numerically correct according to [13] as proposed by the PTB (Braunschweig, Germany).

## RESULTS

While the histological changes that occur after neoplastic transformation in colon cancer are well understood, the changes that occur in the surrounding ECM require investigation. Pursuant to this study, we evaluated the ECM

topography underlying normal, well, and poorly differentiated colon cancer cells. Figure 2 illustrates the histological appearance of normal colonic mucosa, well-differentiated colon cancer, and poorly differentiated colon cancer. Specifically, it shows normal colonocytes with small, regular nuclei and large amounts of cytoplasm in well defined crypts, well-differentiated colon cancer cells in overlapping crypts with larger nuclei, and poorly differentiated colon cancer cells in loose groupings with open faced nuclei. Figure 3 illustrates qualitative data on the ECM underlying normal colonic epithelial cells and colon cancers. It demonstrates that there was a loss of crypts, increase in porosity, and a significant increase in the roughness of the ECM. To make quantitative observations, a computer based SEM-stereoscopy program was used.

For the purpose of this study, the roughness curves provided the greatest amount of information regarding the difference between well-differentiated and poorly differentiated colon cancer extracellular matrix. Specifically, well-differentiated colon cancer ECM topography was more trough-like while poorly differentiated colon cancer ECM was more peak-like, as illustrated in Figure 4. Furthermore, this curve illustrates that characteristics observed were all on the submicrometer scale.

In addition to observing this in graphical form, specific roughness parameters were extracted, as shown in Table 1 [12]. The parameters in Table 1 include the average roughness Ra, which is the integral of the absolute values of the roughness profile, the root-mean square roughness Rq, the maximum height difference Rz of all roughness values, which is the sum of the maximum valley depth Rv, and



**Figure 3:** Scanning electron micrographs of the extracellular matrix underlying normal colonic epithelia (a), well differentiated (b), and poorly differentiated colon cancer cells (c). Tumors processed as described in Materials and Methods, fixed in Trump's fixative, air dried using HMDS, and imaged using a Hitachi S-3000N VP-SEM. Note that the microtopography in (a) is trough-like whereas that in (c) is peak-like. Scale bars = 100  $\mu$ m.

the maximum peak height Rp. The skewness Rsk specifies whether the profile contains many high peaks (Rsk > 0) or many low valleys (Rsk < 0). Additionally we provide the mean absolute slope of the roughness profile Rdq and the RL value which is the ratio between the true length of the roughness profile and the projected length of the roughness profile.

These parameters illustrate that poorly differentiated colon cancers have greater average roughness and steeper mean absolute slopes compared with well differentiated colon cancers. Figure 5 illustrates the truly disordered nature of the ECM of a poorly differentiated colon cancer in 3D and highlights the ability of the software to provide pseudocolor maps with each color corresponding to a specific height. Our data suggest that specific and quantifiable topographical changes occur in colon cancers as they de-differentiate. The physiological consequences of these physical changes in the ECM topography on colon cancer cell behavior remain to be determined.

**CONCLUSIONS**

The scanning electron microscopy stereoscopy program used here has vastly improved our ability to use the SEM as a profilometer in both biological and non-biological disciplines. While the engineering community has had access to contact and optical profilometry, the biological community has been limited in its ability to profile bulky, irregular specimens.

As illustrated above, SEM-S software can be used to gain a novel understanding into how the extracellular matrix in colon cancer changes as a virtue of its state of differentiation. Furthermore, it allows us to quantify roughness parameters that have been previously shown to be important predictors of cell behavior in specimens that were previously difficult to be characterized quantitatively. Thus, SEM-S software packages provide a novel tool for the characterization of biological specimens with scanning electron microscopy.

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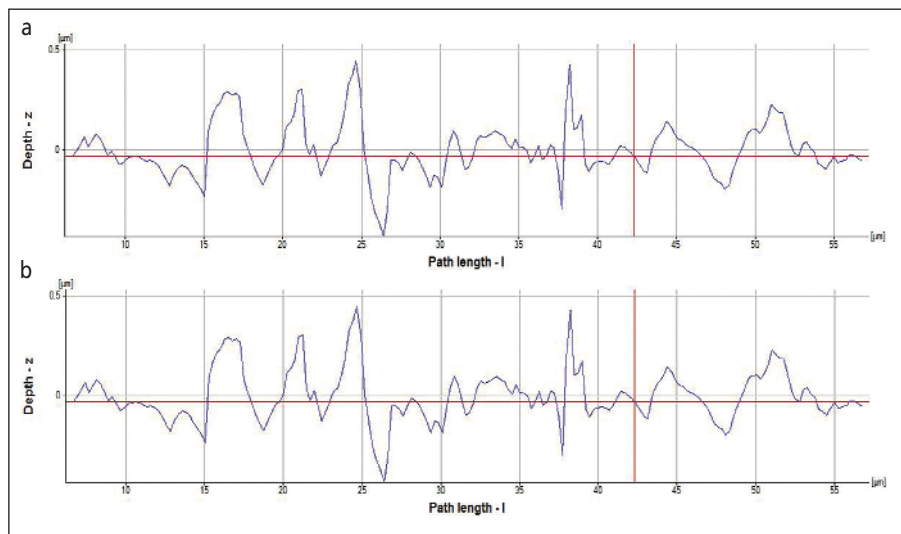


Figure 4: Roughness profiles of well-differentiated colon cancer extracellular matrix (a) and poorly differentiated colon cancer extracellular matrix (b). These profiles illustrate the distinct topographical differences between well-differentiated and poorly differentiated colon cancer ECM. Specifically, well-differentiated colon cancer ECM is more trough-like whereas poorly differentiated colon cancer ECM is more peak-like. These changes in surface topography may have implications in colon cancer cell de-differentiation and spread. Both the x and y axes are in micrometers.

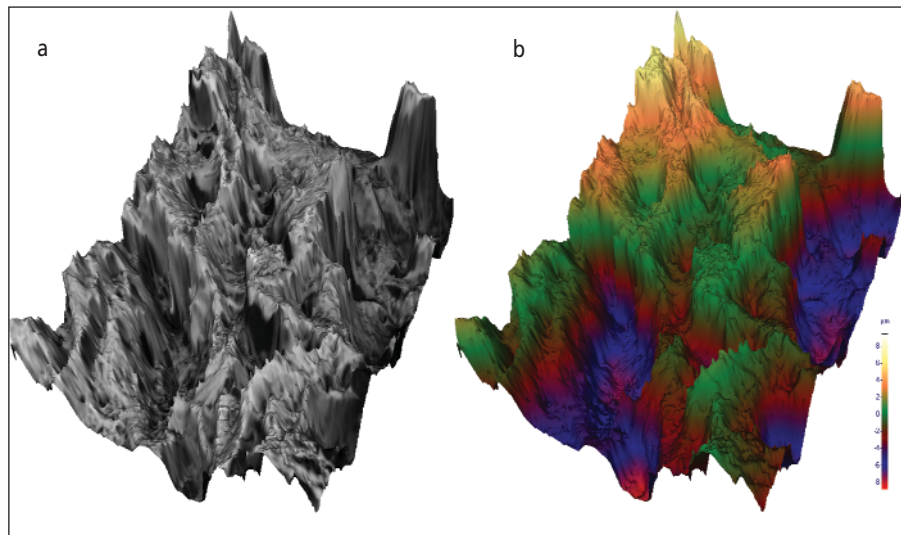


Figure 5: 3D reconstruction of poorly differentiated colon cancer ECM. (a) The 3-D reconstruction in grayscale. (b) The same sample in pseudocolor mode where each color corresponds to a particular height, shown in the scale bar. HFW = 50 μm.

Extracellular Matrix Tissue	Ra	Rq	Rz	Rv	Rp	Rsk	Rdq	RL
Normal Colon	157.3	219.4	2592	1336	1257	-0.339	0.573	1.125
Well Differentiated CC	144.7	203	1925	1017	908	0.187	0.518	1.103
Poorly Differentiated CC	296.9	401.2	2879	1460	1420	-0.238	1.296	1.415

Table 1:

ECM roughness parameters for selected tissues in nm. CC, colon cancer; Ra, mean absolute roughness; Rq, root-mean-square roughness; Rz, maximum profile height; Rv, maximum valley depth; Rp, maximum peak height; Rsk, skewness; Rdq, mean absolute slope of the roughness profile; RL, ratio between the true length of the roughness profile and the projected length of the roughness profile. For exact definitions see [12].

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