

Nano-Architectural Alterations in Mucus Layer Fecal Colonocytes in Field Carcinogenesis: Potential for Screening

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Abstract

Current fecal tests (occult blood, methylation, DNA mutations) target minute amounts of tumor products among a large amount of fecal material and thus have suboptimal performance. Our group has focused on exploiting field carcinogenesis as a modality to amplify the neoplastic signal. Specifically, we have shown that endoscopically normal rectal brushings have striking nano-architectural alterations which are detectable using a novel optical technique, partial wave spectroscopic microscopy (PWS). We therefore wished to translate this approach to a fecal assay. We examined mucus layer fecal colonocytes (MLFC) at preneoplastic and neoplastic time points (confirmed with rat colonoscopy) in the azoxymethane (AOM)-treated rat model and conducted PWS analysis to derive the nano-architectural parameter, disorder strength (Ld). We confirmed these results with studies in a genetic model (the Pirc rat). We showed that MLFC appeared microscopically normal, consistent with field carcinogenesis. Ld was elevated at an early time point (5 weeks post-AOM injection, effect size = 0.40, $P = 0.024$) and plateaued before adenoma formation (10 weeks post-AOM, effect size = 0.66, $P = 0.001$), with no dramatic increase once tumors developed. We replicated these data in the preneoplastic Pirc rat with an effect size in the MLFC that replicated the rectal brushings (increase vs. age-matched controls of 62% vs. 74%, respectively). We provide the first demonstration of a biophotonics approach to fecal assay. Furthermore, targeting the nano-architectural changes of field carcinogenesis rather than the detection of tumor products may provide a novel paradigm for colorectal cancer screening. *Cancer Prev Res*; 6(10); 1111–9. ©2013 AACR.

Introduction

Despite a plethora of widely available tests, colorectal cancer remains the second leading cause of malignancy-related mortality in the United States, underscoring the need for more effective population screening strategies (1). Typically, there is a trade-off between accuracy and patient acceptability/cost in screening tests. For instance, colonoscopy is the "gold standard" for accuracy but is plagued by patient compliance issues (attributable to discomfort, embarrassment, risk of complications, unpleasantness of colonic purge). Further complicating matters is

the juxtaposition of the resource-intensive nature of colonoscopy or other imaging modalities [i.e., computed tomography (CT) colography, capsule endoscopy] versus the remarkably low yield with screen-relevant neoplasia in the at-large population (well under 10%; ref. 2). Therefore, it is clear that "personalizing" risk analysis is essential for more accurate risk stratification, rather than simply designating the majority of the population 50 years and older as being "average risk," which is the current state of the art (2).

In this regard, developing a prescreen using a noninvasive, inexpensive approach would be of great value in determining which "average risk" populations are likely to harbor lesions and thus achieve a survival benefit from colonoscopy (3). One standard approach has been to use fecal assays which generally target tumor-related bleeding via fecal occult blood tests (guaiac or immunohistochemical) or tumor products (e.g., fecal DNA). While these tests have been documented to decrease fatalities from colorectal cancer, the insensitivity to advanced adenomas (and hence cancer prevention) has led them to be relegated to a second-line test in some guidelines (4). Efforts to improve sensitivity using next-generation technologies including DNA mutation analysis, methylation, etc., have been marginally successful (sensitivity for advanced

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adenomas ~50%; ref. 5). Conceptually, fecal DNA assays may be limited by the proverbial "needle in a haystack", as human DNA only accounts for 0.01% of the DNA found in the stool (6). This is compounded further by the shedding of the entire intestinal epithelium every 3 to 7 days which means that only a small fraction of the human DNA would come from the neoplastic lesion.

Therefore, it is of paramount importance to amplify the neoplastic signal. One approach would be to exploit the diffuse alterations associated with field carcinogenesis (also known as field effect or field of injury; ref. 7), thus targeting the much larger non-dysplastic mucosa (8). Field carcinogenesis is a well-established biologic concept that provides the underpinning for several aspects of current clinical practice. For example, identification of an adenoma mandates assessment for concurrent (synchronous) lesions (full colonoscopy if a polyp is found on flexible sigmoidoscopy; ref. 9) and future (metachronous) lesions (follow-up colonoscopy at more frequent intervals given the high risk of recurrent lesions; ref. 10). As it is clear that adenoma appears to be a relatively insensitive marker for field carcinogenesis, there has been interest in assessing biomarkers that occur at an earlier stage (i.e., microscopically normal mucosa). Attention has focused on a variety of molecular markers (methylation, genomics, proteomics, etc.; ref. 11) that have been shown to be altered in the endoscopically normal distal colonic mucosa, although the performance/practicality of these assessments presents difficulties in translating to clinical practice (8).

Our group has developed partial wave spectroscopic microscopy (PWS), an optical technology that evaluates the nano-architectural consequences of the subtle genetic/epigenetic changes in field carcinogenesis (12). Specifically, PWS is sensitive to structures at 10 to 100 nm, thereby allowing quantification of the fundamental cellular "building blocks" (nucleosomes, ribosomes, macromolecular complexes, etc.) that have been implicated in early carcinogenesis (12).

We have previously shown that PWS analysis showed profound nanocytological changes [quantified by the parameter disorder strength (Ld)] from the microscopically normal rectal mucosal brushings in patients harboring neoplasia elsewhere in the colon (13). Furthermore, the magnitude of rectal Ld changes mirrored the colorectal cancer risk. However, for optimal clinical implementation, adaptation to a fecal test would be required. The major obstacle to date has been that the vast majority of fecal colonocytes are apoptotic and thus unsuitable for micro-architectural analysis. Recently, however, mucus layer fecal colonocytes (MLFC) have been isolated which appear to be morphologically well preserved (nonapoptotic; ref. 14). We therefore wanted to determine whether PWS analysis of MLFCs could predict risk of colorectal cancer at preneoplastic time points (recapitulating field carcinogenesis). For these studies, we used a well-validated model of colorectal cancer, a carcinogen model [the azoxymethane (AOM)-treated rat] which was complemented by a genetic model (the Pirc rat; ref. 15).

Materials and Methods

Animal studies

All studies were conducted under the auspices and supervision of the Institutional Animal Care and Use Committee of Northshore University HealthSystem (Evanston, IL).

AOM-treated rat. Male Fisher 344 Rats were obtained at 7 to 8 weeks of age and fed the AIN 76-A diet (Harlan Teklad). Rodents were randomized with 2 weekly intraperitoneal injections of either AOM (15 mg/kg of weight) or saline (Harlan Teklad). We first wanted to determine whether the premalignant colonocytes would manifest alterations in Ld. The AOM-treated rat has a well-defined time line with adenomas requiring about 15 to 20 weeks to develop and carcinomas about 35 to 40 weeks. We looked at the earliest time points (4–8 weeks post-AOM) by which time the nonspecific (toxic) effects of the carcinogen have dissipated (16, 17).

Pirc (polyposis in rat colon) rat. Twenty Pirc rats were obtained from Taconic Farms: These rats contain a germ line mutation in the adenomatous polyposis coli (APC) tumor suppressor at codon 1137 leading to the development of multiple colonic neoplasms in about 3 to 4 months (15).

Rat colonoscopy. Serial colonoscopic evaluation was conducted via rigid rat colonoscope (Coloview; Karl Storz). Overnight fasted rats were sedated (with isoflurane), secured in the supine position, and a well-lubricated colonoscopic probe introduced slowly via anus with gentle air insufflation. The tumor images were captured during the probe withdrawal using the Image 1 camera system (Karl Storz).

Fecal colonocyte isolation

This was conducted by modification of a protocol by White and colleagues (14). Fresh stool was collected within 2 hours of evacuation. Stool was processed from AOM and saline groups as follows: An aliquot of stool was placed in a plastic bag and washed in PBS 1×. The sample was then agitated in 0.05% ammonium thioglycolate/PBS wash at a ratio of 2 mL/g of stool (Sigma-Aldrich) and pressed to liberate mucus from the outer fecal pellet. Five milliliters of thioglycolate wash was added to the sample and centrifuged at 1,000 rpm for 5 minutes at 4°C. Samples were then decanted and re-suspended in CytoPreserv solution (Hologic) at a ratio of 5 mL/g of original stool. Samples were incubated at 4°C for 1 hour. Samples were then filtered through a 300-µm WhirlPak Bag (Nasco) to remove large debris. Samples were further filtered through a 125-µm mesh (Small Parts, Inc.) to collect the fecal mucus layer. Samples in the mesh were collected in 30 mL of CytoPreserv solution and then centrifuged at 800 rpm for 5 minutes at 4°C, forming a thin mucus layer above the pellet. Mucus layer was removed and incubated in a combination of 1.5 mmol/L EDTA and 0.5 mol/L of *N*-acetyl L-cysteine (Sigma-Aldrich) for 10 minutes at 37°C. Samples were then added to CytoLyt solution (Hologic) to dilute mucolytic agents and fixed onto glass slides using ThinPrep 2000 (Hologic).

PWS analysis

The PWS instrument used for this study has been previously described (12, 18). PWS measures the disorder

strength of intracellular architecture using the parameter (L_d). $L_d = L_C \delta n^2$, where δn^2 is the variance of the spatial refractive index fluctuations. In short, for a given specimen, the PWS system generates a 3-dimensional (3-D) data cube termed as the fluctuating part of the reflection coefficient where (x, y) refer to a specific pixel in the object plane and λ is the wavelength. For a given unstained cell, the spectral fluctuations are calculated in the wavelength range of 500 to 700 nm (above the system noise floor) by means of 1-D mesoscopic light transport theory to obtain L_d . Thus, a map of disorder strength is obtained from each pixel (x, y) . Using this 2-D map, for each cell, the mean intracellular disorder strength (the average over x and y pixels) is obtained. The average for a group of cells (~ 20 – 30 cells for each time point) is calculated and defined as the mean disorder strength per time point. The L_d average and the SE are depicted in all the histograms in this report. We note that slide-to-slide variability for each time point was negligible ($P > 0.10$). All of the PWS analysis was conducted by an investigator who was blinded to origin of cells/tissue (saline-treated vs. AOM or wild-type vs. Pirc rat).

Rectal brushings

Rectal cells from the visually normal mucosa of the Pirc and age-matched wild-type rats were obtained with a cytology brush. The brush was gently applied to a microscope slide, fixed with 70% ethanol, air-dried, and then subjected to PWS analysis.

5-Bromo-3-deoxyuridine pulse-chase experiments

To determine whether isolated fecal colonocytes were obtained through epithelial abrasion from stool passage, rats were injected with 50 mg/kg of 5-bromo-3-deoxyuridine (BrdUrd; Sigma-Aldrich) dissolved in sterile PBS. BrdUrd is a thymidine analogue which is incorporated into proliferating cells. After incorporation, colonocytes migrate up the crypt in a time-dependent fashion (19). After 48 hours, fresh stool was collected and animals were euthanized. Four-micrometer distal rat colon section and fecal colonocyte slides underwent BrdUrd immunostaining with a biotinylated mouse anti-BrdUrd antibody (Invitrogen.) as previously described (20).

Statistical methods

All statistical analysis was conducted using Microsoft Excel (Microsoft Corporation). A standard 2-tailed t test (assuming unequal variances) with $P \leq 0.05$ considered statistically

$$\text{Effect size} = \frac{\mu_1 - \mu_2}{\sqrt{\sigma_1^2 + \sigma_2^2}}$$

where μ_1 and μ_2 are the means for the AOM-treated or Pirc models and control groups, respectively, and σ_1 and σ_2 are the corresponding SDs. We used this effect size definition to take into account the slide-to-slide variability and to robustly measure the statistical significance of the average

L_d difference, that is, for control and carcinogen-treated or Pirc rat.

Results

Experimental models

We used both carcinogen and genetic experimental models in these studies given the time frame for neoplastic development was well-characterized and the animals could be followed longitudinally with fecal sampling at various time points. We used a rigid colonoscope to determine the tumor-bearing status in the distal 80% of the colon where the vast majority of tumors are located.

The ability to visualize the colon *in vivo* was excellent. The age-matched control (saline injected) rat was tumor free (Fig. 1A), whereas Fig. 1B shows representative images with the AOM-treated rat indicating distinct tumors at 24 weeks. Similarly, the age-matched wild-type rats were tumor free (Fig. 1C), whereas the Pirc rat colon showed the characteristic multiple adenomas (polyposis; ref. Fig. 1D).

MLFCs: isolation and characterization

Stool was recovered immediately before rat colonoscopy. With regard to fecal colonocytes, our yield was about 15 cells/g of stool. As our previous works indicated that PWS analysis of field carcinogenesis necessitates about 30 to 50 cells, we used about 2 to 4 g of stool aliquots for this analysis (13, 21). Review by pathologist (S.E. Crawford) confirmed that the majority of cells ($\sim 80\%$) was morphologically preserved (nonapoptotic) colonocytes (Fig. 2A) with the remainder being squamous (presumably anal in origin) or scattered inflammatory cells (macrophages, etc). To further support the notion that the colonocytes were abraded by stool bolus rather than sloughed by apoptosis, we wanted to analyze the location of these cells in regard to the crypt. We reasoned that as apoptotic cells are sloughed from the tip of the crypt, if we were able to show that some of the cells were at the base of the crypt, this would strongly argue against apoptosis (22). To label the cells, we injected animals with BrdUrd which is incorporated into proliferating cells which reside in the base of the crypt. Typically, these labeled cells migrate toward the top of the crypt as they mature (~ 5 – 7 days; ref. 19). We confirmed 2 days postinjection [Fig. 2B (i)] that all the labeled colonocytes (specifically nuclei as BrdUrd is incorporated into DNA) were confined to the bottom half of the crypt (there was some nonspecific cytoplasmic blush toward the luminal surface). Importantly, we were able to note in the stool recovered that a small proportion ($\sim 3\%$ – 5%) of the MLFCs had evidence of BrdUrd uptake, suggesting that they were not from the apoptotic areas [tip of the crypt; Fig. 2B (ii)].

PWS analysis of fecal colonocytes from AOM-treated rat model

First, we investigated the ability of PWS to differentiate microscopically normal fecal colonocytes isolated from the predysplastic AOM-treated rats ($n = 4$) versus age-matched saline ($n = 4$). We used the AOM-treated rat because it is

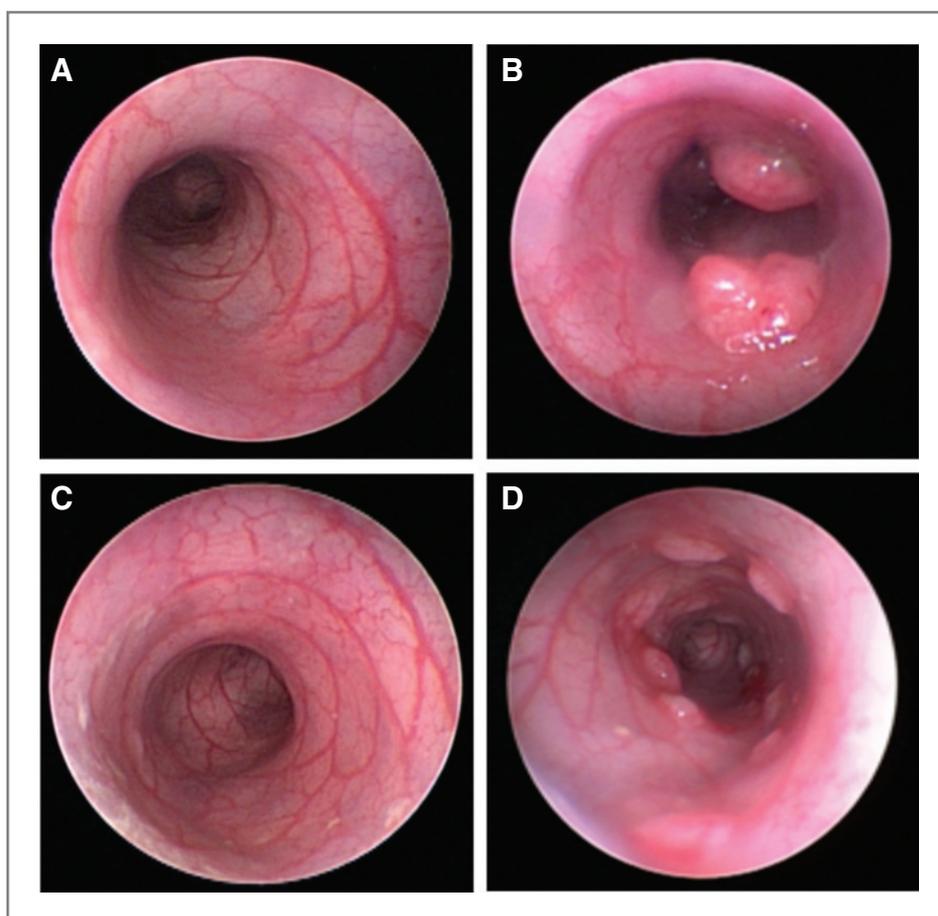


Figure 1. Representative colonoscopic images from animal models of experimental colon carcinogenesis. For these longitudinal studies, rat colonoscopy was conducted to evaluate tumorigenesis status of animals at the time point of fecal sampling of AOM-treated rat without and with tumor. A, twenty-four-week-old Fisher 344 controls (saline-treated) without tumors. B, twenty-four-week-old AOM-treated Fisher 344 rats with adenomas. C, APC wild-type rat colon (14 weeks old)—no tumor noted. D, Pirc rat—14 weeks old with evidence of polyposis with numerous adenomas detectable.

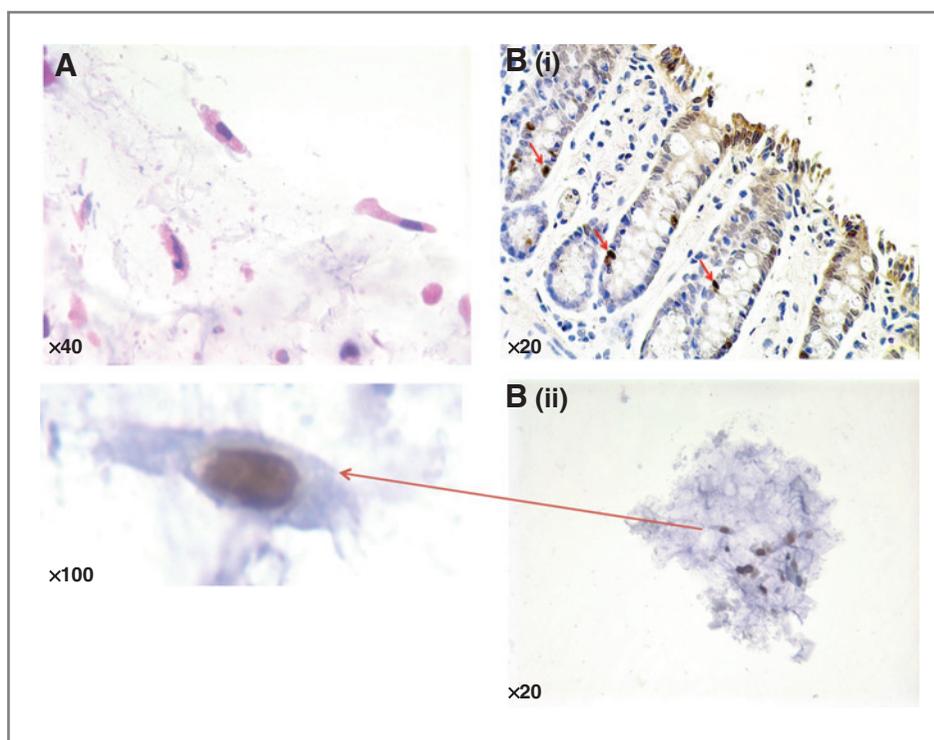
well-validated (the leading model of experimental colonic neoplasia over the last several decades) and the neoplastic timeline is well-established (i.e., aberrant crypt foci, adenomas, and carcinomas: ~5, ~20, and ~35–40 weeks, respectively; refs. 16, 17). Furthermore, this model allows ready longitudinal analysis for tumors via rat colonoscopy. No time points before 2 weeks were evaluated, as it has been shown that there may be nonspecific carcinogen effects; however, this dissipates within 10 days after carcinogen treatment (16).

As previously noted, fecal colonocytes appeared microscopically identical suggesting that structures at length scales of above 200 to 500 nm (diffraction limit of light) were largely unchanged (Fig. 3A). However, at smaller length scales of PWS analysis (>10–20 nm), there were marked differences. Indeed, when a disorder strength (Ld) pseudocolor map was superimposed on the images, there appeared to be a marked increase in Ld in the fecal colonocytes from AOM-treated animals (denoted by red color coded regions) when compared to the age-matched saline-treated controls. (Fig. 3A) The Ld appeared to be altered between AOM and saline throughout the cell, consonant with our previous work which had noted that differential signals occurred both in the nucleus (high-order chromatin) and cytoplasm (driven at least partially by cytoskeletal alterations; ref. 23).

We then quantified the mean fecal colonocyte Ld at various time points during carcinogenesis (Fig. 3B and C). When compared to age-matched controls, there was no difference in disorder strength (ΔLd) at 2 weeks post-AOM treatment (effect size = 0.052, $P = 0.82$), whereas all other time points manifested a significant difference. The SDs were large presumably related to the somewhat skewed Ld distribution in both the saline- and AOM-treated colonocytes. For instance, ΔLd increased significantly (effect size = 0.40, $P = 0.024$) at 5 weeks after the AOM treatment. The differential became more pronounced at week 8 (effect size = 0.47, $P = 0.023$) and was maximal after 10 (effect size = 0.66, $P = 0.001$) and 18 weeks (effect size = 0.70, $P = 0.0017$) post-AOM treatment. Importantly, previous results have suggested that this time point may best replicate human field carcinogenesis (16).

It needs to be emphasized that these alterations in nanoscale structure in carcinogenesis reflect field changes and occurred before tumorigenesis and thus were not confounded by tumor-related fecal colonocytes. While the focus of our studies was mainly on premalignant time points, the 32-week animals did have evidence of tumors. The salient finding at this time point was that while the fecal colonocytes still manifested a highly diagnostic elevation in Ld, this was equivalent to earlier times (weeks 15 and 20),

Figure 2. MLFC isolation and characterization. A, representative fecal colonocytes showing well-reserved columnar. B, BrdUrd chase experiments. (i), section of colon stained with BrdUrd (20 \times magnification) – 14-week-old AOM-treated rats 48 hours postinjection where positive nuclei are confined to the bottom half of the crypts. As BrdUrd is incorporated in the nucleus, the cytoplasmic blush at the luminal surface is artifactual. (ii), MLFCs obtained from these same animals immediately before tissue biopsy.



suggesting that the tumor *per se* was not adding perceptibly to Ld from the MLFCs.

Results for Pirc rats rectal brushing

We next wanted to show that the fecal colonocyte approach was not model-specific. Therefore, we selected a genetic model, the Pirc rat (polyposis in rat colon) to complement the carcinogen rat study. The advantage of this model is that given its germ line mutation in the APC tumor suppressor gene, it recapitulates the genetic initiation of most sporadic human colorectal cancers. To show that PWS was effective at identifying field carcinogenesis in this model, we used rectal brushings at a time point before adenoma development (8 weeks of age, pre-adenoma status confirmed by rat colonoscopy). These rectal brushings ($n = 5$ wild-type vs. $n = 7$ Pirc) were applied to a glass slide and then underwent PWS analysis on the colonocytes. As indicated in Fig. 4A, there was a 74% increase in Δ Ld (effect size = 0.47, $P = 0.0005$) between the Pirc and age-matched APC wild-type rats. When we analyzed MLFCs (Fig. 4B) from corresponding animals (8 weeks of age), we found that the Δ Ld was increased 62% (effect size = 0.39, $P = 0.03$). The absolute Ld values were somewhat altered between brushing and fecal colonocytes but may relate to the different fixation techniques. Importantly, the similarities between the rectal brushing and fecal colonocyte effect size data provide further corroboration that MLFCs are actually being obtained from the normal (nonapoptotic) epithelium. Hence, the effect observed by PWS analysis conducted on the MLFCs is not limited by the animal model but could be a universal phenomenon.

Discussion

We report herein that PWS analysis of MLFCs was able to detect the early nano-architectural signatures of field carcinogenesis and hence identify risk of colonic neoplasia. We show this in two well-validated models: a carcinogen and a genetic model of colorectal cancer. Importantly, we focused on premalignant mucosa (colonoscopically confirmed), thus replicating the potential clinical applications. This is the first demonstration that optical techniques can be used as fecal assays. Furthermore, it shows the power of PWS to practically detect nano-architectural aberrations in cytologically normal epithelial cells.

The approach of identifying field carcinogenesis rather than tumor products/tumor-related bleeding provides a different paradigm for fecal assays. Colon field carcinogenesis can be identified by morphologic characteristics (adenomas or aberrant crypt foci) or biomarkers from the microscopically normal mucosa such as cellular (apoptosis/proliferation; ref. 24) and molecular (genomic, proteomic, microRNA, methylation, etc.) markers (25–28). However, all of these require instrumenting the colon, presenting a hurdle to patient uptake. Stool studies can overcome these obstacles, and several stool biomarkers have been shown to be altered in field carcinogenesis (vimentin methylation or microRNAs such as hsa-miR-342 or miR-137; refs. 29, 30).

Nano-architectural biomarkers are particularly attractive because they may represent a common pathway for a myriad of preneoplastic genetic/epigenetic events. Our previous work has indicated that PWS is exquisitely sensitive to subtle alterations in expression of tumor suppressor genes

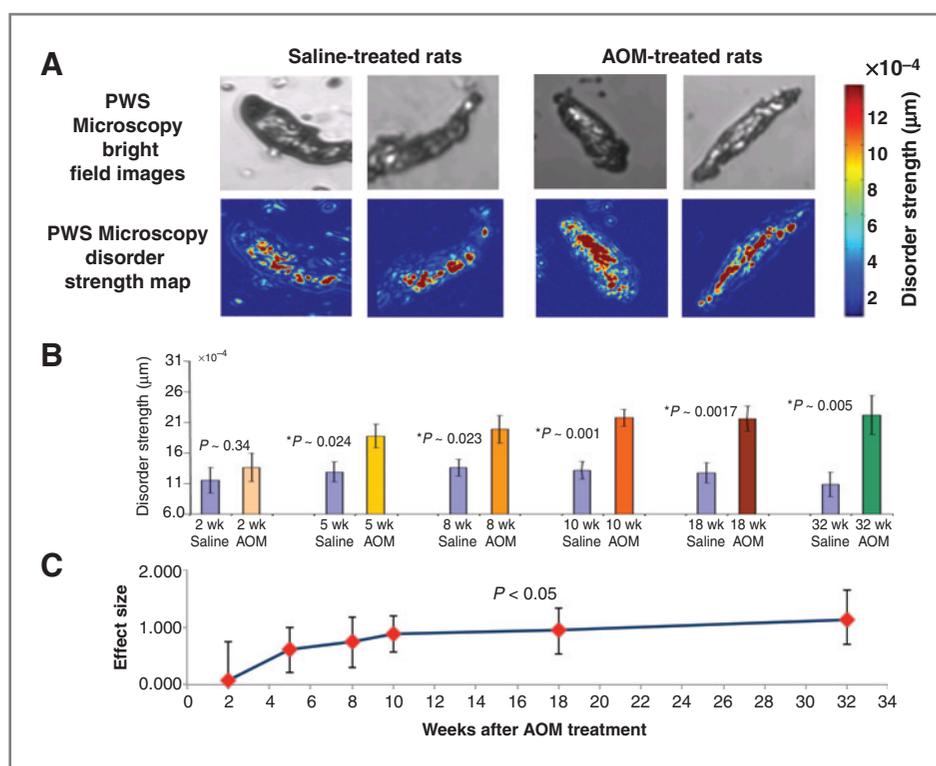


Figure 3. PWS analysis of AOM-treated rat. A, PWS analysis of fecal colonocytes: using the bright-field capability of the PWS microscope, images of unstained fecal colonocytes from both AOM-treated (at pretumorigenic time points) and control animals were taken and appeared identical. However, using Ld, pseudocolor map was markedly different with higher Ld areas in the AOM-treated rat being noted in both the nucleus and cytosol. B, Ld quantification: as the saline-treated animal fecal colonocytes Ld did not change over time (see supplementary Fig.), all time points were collectively used as comparator. With regards to AOM treatment, there was an early rapid stepwise induction in mean cellular Ld with significant differences noted at 5 weeks and plateauing ~ 10 weeks post-carcinogen. C, effect size: to gain insights into the diagnostic performance, we examined "effect size" of mean cellular Ld (vs. saline control) and was plotted with 95% confidence intervals. Similar to the absolute differences, the effect size was significantly increased at 5 weeks and the magnitude appeared to plateau ~ 10 weeks post-carcinogen treatment.

and proto-oncogenes, with Ld correlating well with a more malignant phenotype in microscopically identical cells (12). We also noted that in the MIN mouse (the murine equivalent of the Pirc rat), the biophotonic analysis of

brushings at a preneoplastic time point accurately discriminated between animals with and without the APC mutation (31). We have shown in human studies that brushings of the endoscopically normal mucosa showed a progressive

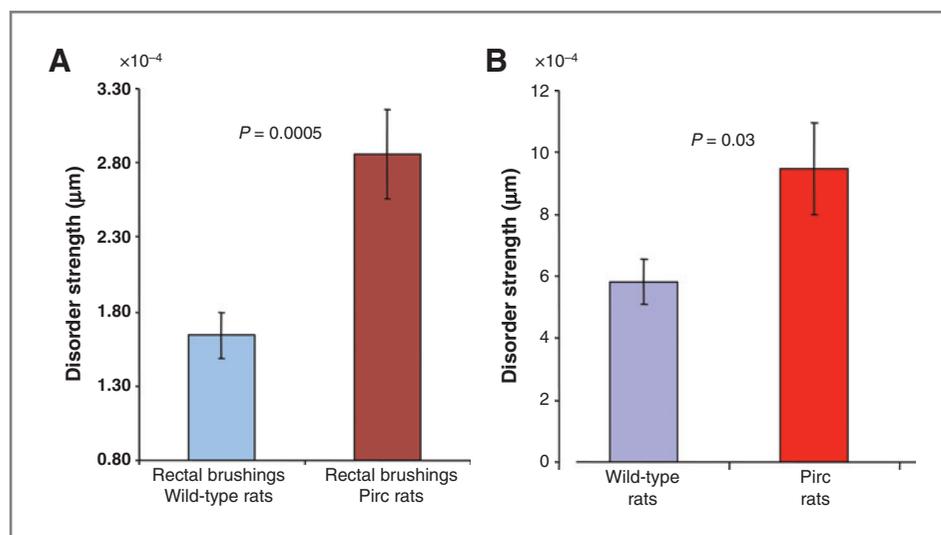


Figure 4. PWS analysis of Pirc rats. A, rectal brushings—PWS-measured Ld was significantly higher (74% increase, $P = 0.0005$) for isolated rectal colonocytes obtained from 8-week-old Pirc rats ($n = 7$ animals) compared with age-matched APC wild-type rats ($n = 5$ animals). B, fecal colonocytes: PWS results from fecal colonocytes of Pirc rats—Ld was significantly higher (62% increase, $P = 0.03$) for isolated fecal colonocytes obtained from 8-week-old Pirc rats compared with age-matched APC wild-type rats.

induction of Ld in patients with nonadvanced adenomas, advanced adenomas, and carcinomas (~125%, ~225%, and ~400% of control, respectively; ref. 13). Thus, nanocytology represents a powerful modality for field effect identification.

Translation of PWS to fecal colonocyte analysis has to overcome an obstacle in that most fecal colonocytes shed via apoptotic and are thus unlikely to retain informative ultrastructural data. Several lines of evidence support our thesis that MLFCs are structurally normal colonocytes and thus can represent field carcinogenesis. For instance, our data (consistent with others) have shown that they were generally morphologically preserved (32). Moreover, the finding that some MLFC showed BrdUrd incorporation (at a time point when tissue BrdUrd-positive cells was confined to the bottom half of the crypt) suggests nonapoptotic mechanisms for extrusion of these cells in the colonic lumen. This provides strong support for the notion that these cells are normal epitheliums which are abraded by the passage of a formed stool bolus. In this regard, it is interesting to note that the effect size was equivalent between the rectal brushings and fecal colonocytes, further corroborating the abrasion concept.

The ability to sense the nanoscale correlates of field carcinogenesis is a testament to the power of PWS for sensing subdiffractional length scales and cellular structure. Because optical refractive index is a linear function of the local density of intracellular solids (proteins, lipids, DNA, and RNA), the spectrum of a scattering signal contains information about spatial variations of density at length scales that are well below the wavelength. This is the main principle of PWS, which is capable of extracting 1-D propagating waves from different parts of a scattering particle such as a cell. Our previous studies have shown that in realistic experimental conditions, the limit of PWS sensitivity to structures is under 20 nm (33). Thus, PWS opens up the realm of cellular nanostructure for quantification.

The identity of the structures that lead to the altered Ld in fecal colonocytes has yet to be elucidated. Our previous reports indicate that the signal can emanate from both the nucleus and cytoplasm. However, there are myriad candidate structures at this length scale that are integral to colon carcinogenesis including mitochondria/ribosomes and high-order chromatin (23, 34).

In addition to the technological breakthrough of PWS, another innovation lies in clinical application. Most studies on novel optical technologies have largely focused on the optical biopsy—identifying the histology of lesions *in situ* which can be quite important in the application of endoscopy (35). Our focus has been on risk stratification via field carcinogenesis detection in a variety of organs including colon, lung, pancreas, ovary, and esophagus (18, 21, 34). Our previous work showed that PWS nanocytology from the rectum was able to predict both concurrent and future neoplasia in patients via brushings of the endoscopically normal rectal mucosa (13). The clinical imperative for colorectal cancer risk stratification is that the vast majority

of screening colonoscopies (>90%) are unproductive from a cancer prevention perspective, whereas much of the population lacks access to these finite endoscopic resources. The current strategy of grouping all patients ≥ 50 years of age without a personal and family history of colonic neoplasia as "average risk" is biologically naïve and leads to inefficiency. Attempts to stratify based on standard colorectal cancer risk factors (family history, diet, obesity, tobacco, etc.) yielded an improvement but still offer fairly modest predictive ability (AUROC of NIH risk score for colorectal cancer is 0.60; ref. 36). We speculate that fecal PWS may have the requisite clinical performance based on the power of the technology and because field carcinogenesis is impacted by both genetic and exogenous factors.

Furthermore, a two-step process (noninvasive pre-screen, with positive results being offered the more definitive colonoscopy) is the basis of current approaches such as flexible sigmoidoscopy, CT colography, and the widely used fecal approaches such as the fecal immunohistochemical test (FIT). Fecal tests are particularly attractive given their low cost and minimal intrusiveness. It needs to be highlighted that improved compliance engendered by fecal tests can ameliorate differences in accuracy as shown by the equivalent colorectal cancer yield of FIT and colonoscopy in a recent study. Thus, as this early report appears to suggest that field carcinogenesis detection with fecal PWS has considerable promise, the future potential public health implications for this approach may be substantial.

There are several limitations to this work that need to be acknowledged. First, it exclusively involves animal models such that the translatability to humans needs to be confirmed. On the other hand, the demonstration in both a well-validated carcinogen and a genetic model ameliorates this concern. Furthermore, we have previously published that PWS is highly accurate at detecting field carcinogenesis in humans via rectal brushings (13). Second, the issue of whether high-quality MLFCs will be recoverable in humans needs to be addressed. While our studies were exclusively on rats, White and colleagues have reported copious colonocytes recovered in human feces by a similar protocol (14). Third, the effect of concurrent neoplasia was not rigorously explored, although the thrust of this approach is to evaluate risk and, if anything, one would expect either an enhanced or unaffected diagnostic performance (consistent with our 32-week AOM rat data).

In conclusion, our report provides the proof of principle that fecal colonocyte analysis can be used for field carcinogenesis detection which can be identified using a powerful new biophotonics approach, PWS. This is the first report on using biophotonics for fecal colonocyte analysis and thus the uncoupling of gastrointestinal (GI) tract optical applications from endoscopy. This approach may herald a change in fecal tests from simple detection of minute quantities of neoplastic products in the fecal stream to the more amplified field carcinogenesis signal, thus enabling the prediction of both current and future risk. From a clinical perspective, fecal PWS analysis may serve as a

prescreen to identify both patients likely to benefit and, equally importantly, those who can safely eschew colonoscopy. This approach could be a paradigm shift, allowing for the personalization of colorectal cancer screening.

Disclosure of Potential Conflicts of Interest

H.K. Roy, H. Subramanian, and V. Backman are cofounders and shareholders in Nanocytomics LLC. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: H.K. Roy, D. Damania, M. DelaCruz, D.P. Kunte, H. Subramanian, R.K. Wali, V. Backman

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.K. Roy, D. Damania, A.K. Tiwari

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Damania, S.E. Crawford, A.K. Tiwari, R.K. Wali, V. Backman

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.K. Roy, M. DelaCruz, A.K. Tiwari

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