Early Prediction of Cancer Progression by Depth-Resolved Nanoscale Mapping of Nuclear Architecture from Unstained Tissue Specimens

Shikhar Uttam, Hoa V. Pham, Justin LaFace, Brian Leibowitz, Jian Yu, Randall E. Brand, Douglas J. Hartman, and Yang Liu

Abstract

Early cancer detection currently relies on screening the entire at-risk population, with colonoscopy and mammography. Therefore, frequent, invasive surveillance of patients at risk for developing cancer carries financial, physical, and emotional burdens because clinicians lack tools to accurately predict which patients will actually progress into malignancy. Here, we present a new method to predict cancer progression risk via nanoscale nuclear architecture mapping (nanoNAM) of unstained tissue sections based on the intrinsic density alteration of nuclear structure rather than the amount of stain uptake. We demonstrate that nanoNAM detects a gradual increase in the density alteration of nuclear architecture during malignant transformation in animal models of colon carcinogenesis and in human patients with ulcerative colitis, even in tissue that appears histologically normal according to pathologists. We evaluated the ability of nanoNAM to predict “future” cancer progression in patients with ulcerative colitis who did and did not develop colon cancer up to 13 years after their initial colonoscopy. NanoNAM of the initial biopsies correctly classified 12 of 15 patients who eventually developed colon cancer and 15 of 18 who did not, with an overall accuracy of 85%. Taken together, our findings demonstrate great potential for nanoNAM in predicting cancer progression risk and suggest that further validation in a multicenter study with larger cohorts may eventually advance this method to become a routine clinical test.

Introduction

One of the greatest challenges for early cancer detection is how to effectively manage patients who are at risk for developing invasive cancer, such as patients with ulcerative colitis or those with precancerous lesions. Most at-risk patients will not develop cancer and do not warrant treatment or frequent surveillance. Without an accurate means to assess which at-risk patients are likely to progress into malignancy at an early stage of cancer development prior to the detection of clinically significant lesions, clinicians and patients may opt to pursue additional invasive procedures for surveillance or treatment; conversely, a more conservative approach could miss the opportunity to address cancer at an early stage.

Cancer arises mostly from accumulation of somatic mutations, which are further regulated by epigenetic changes that transform normal cells into malignant form. Nuclear architecture, organized into condensed and open compartments (1), plays an important role in regulating the function of genome and epigenome during cancer progression (2, 3). Current clinical gold standard for diagnosing cancer and predicting cancer progression risk relies on the evaluation of morphologic features of stained tissue slide from formalin-fixed, paraffin-embedded (FFPE) tissue section by a trained pathologist using bright-field microscope. The visible microscopic changes in cell nuclei are based on spatial distribution of light absorbance by DNA-binding dyes, such as hematoxylin and Feulgen stains. For example, coarse aggregation of condensed chromatin, hyperchromasia, and nuclear pleomorphism (4–8) are among the established nuclear architecture characteristics of cancer cells. Despite its clinical significance, conventional microscopic nuclear architecture does not provide enough sensitivity to predict cancer progression risk on a personalized level. Conventional approach to assess nuclear architecture uses nuclear stains as surrogate marker, and their ability to reflect the underlying architecture depends on the affinity of the dye and its chemical interaction with the nuclear component. Subtle change in nuclear density may not be detectable with conventional light microscopy.

We have developed a new method to image nuclear architecture on unstained tissue by quantifying the intrinsic depth-resolved density alteration of nuclear architecture, referred to as nanoscale nuclear architecture mapping (nanoNAM). The image resolution of nuclear architecture map is still diffraction-limited, but the image contrast with nanoscale precision is produced by depth-
resolved optical path-length difference (drOPD) arising from the alteration of three-dimensional refractive index profile within the nucleus. We refined nanoNAM for use with standard unstained deparaffinized tissue slide made from FFPE tissue block to account for variations in clinical sample preparation, such as tissue section thickness and staining.

As a proof-of-concept, we first applied nanoNAM to unstained tissue slide from an animal model in which cancer is induced by inflammation agent and carcinogen. To evaluate the clinical utility of nanoNAM, we examined tissue from patients with ulcerative colitis, a disease characterized by chronic colonic inflammation and carrying increased risk for high-grade dysplasia (HGD) and colorectal adenocarcinoma. Clinical guidelines currently recommend that these patients undergo annual or biennial colonoscopy with 4-quadrant biopsies every 10 cm throughout the involved colon (9, 10); thus, a tool to better distinguish those at genuine risk for cancer from those in whom surveillance could be relaxed would improve outcome, cost effectiveness, and patient’s quality of life. Here, we describe our technique for mapping nuclear architecture at a nanoscale sensitivity and present results in animal and human tissue.

Materials and Methods

Depth-resolved nanoNAM

The depth-resolved nanoNAM is essentially a derivative of spectral-domain optical coherence tomography. It has been shown that the phase, rather than the amplitude, of Fourier-transformed spectral interference signal captures the subresolution structural changes in optical path length at a strong interface of interest (11–15), where the refractive index between the tissue and the surrounding medium has a strong mismatch. However, when analyzing nuclear architecture from FFPE tissue section, such nanoscale sensitivity is often compromised because the tissue section thickness cannot be controlled with a nanometer precision. We overcome this limitation by constructing a tissue slide with closely matched refractive index between the tissue and mounting medium. In the absence of a strong interface, the phase of Fourier-transformed spectral interference signals at each of the predefined optical-depth locations quantifies nanoscale structural characteristics within the coherence-gated optical-depth location (16), referred to as depth-resolved OPD (drOPD). Illustration of this concept by numerical simulation and experimental validation is shown in Supplementary Methods and Supplementary Figs. S1 to S3.

To enable drOPD imaging on the glass slide–based tissue samples, a reflection-mode common-path spectral interferometer is built upon the glass slide–based sample itself (Fig. 1A1). The reflection from the single-layer dielectric coating (~20% reflection and 80% transmission; Abrisa Technologies) on the standard glass slide generates a stable reference wave. The tissue or cell section is placed on the coated side of the slide, deparaffinized and mounted with mounting medium to match the refractive index of the sample and minimize the strong interface, and then covered-slipped (Supplementary Methods). The backscattered waves from the tissue, together with the reference waves, are collected as a function of wavelengths (i.e., spectral interference signal).

Due to the lack of sufficient image contrast to reliably identify cell nuclei in drOPD and transmission phase images, we developed an optical microscopy system that consists of three complementary imaging modules to utilize the strength of each for the entire tasks of nanoNAM, as shown in Fig. 1A2: (i) reflection-mode low-coherence spectral interferometry for drOPD mapping of unstained tissue; (ii) transmission quantitative phase imaging of unstained and stained [with hematoxylin and eosin (H&E)] tissue to obtain a high-contrast transmission phase map for identifying overall tissue architecture of unstained tissue, which also serves as an image registration reference; (iii) bright-field imaging of H&E-stained tissue for nuclei identification and pathology correlation. The details of instrument and the workflow for nanoNAM are in Supplementary Methods and Supplementary Fig. S4. To reliably identify the cell nuclei on the low-contrast drOPD map of unstained tissue, we first co-register the transmission phase images of unstained and stained tissue by computing the affine transformation between them based on their similar image features and contrast (Supplementary Methods and Supplementary Fig. S7), which is then applied to bright-field image of stained tissue to register it with drOPD image of unstained tissue. Nuclei segmentation (Supplementary Methods) is performed on the registered bright-field image of the stained tissue to generate nuclei mask that is applied to the drOPD image of unstained tissue at each depth for depth-resolved nanoNAM.

Figure 1B shows the output of nanoNAM and complementary characteristics of the three imaging modalities: Fig. 1B1, the bright-field image of H&E-stained tissue, as the standard image for pathology evaluation, provides clear identification of cell nuclei, but the image contrast of nuclear architecture is generated by the absorption of nuclear stains integrated along the entire tissue thickness, without sufficient details of internal architecture (Fig. 1B1ii); Fig. 1B2, transmission phase image, provides high-contrast overall tissue architecture visualization for unstained tissue such as epithelium and stroma, but the nuclear architecture map comes from the integrated optical path length (OPL) through the entire tissue thickness and the individual epithelial cell nuclei cannot be unambiguously identified (Fig. 1B2ii); Fig. 1B3, the depth-resolved nuclear architecture map of unstained tissue, derived from nanoNAM of segmented epithelial cell nuclei, reveals distinct patterns at three different depths (Fig. 1B3ai–1B3ii; Supplementary Movie S1) that are not visible in Fig. 1B1–1B2. Nuclear architecture is quantitatively described by the nanoscale drOPD value, as shown in the color bar. The deeper red color represents higher drOPD, indicating higher change of refractive index or denser alteration in nuclear architecture. The reproducibility of drOPD value at a single-nucleus level over one-week repeated measurement with different acquisition time is 1 to 2 nm (Supplementary Methods and Supplementary Fig. S10).

Calculation of drOPD map

We obtained the spectral interference signal P(k) (see Supplementary Methods for more detailed mathematical explanation) from the sample area \(\langle I_{\text{sample}}(x, y, k) \rangle\) and the reference signal \(R_s\) on a background area (no sample) \(\langle I_{\text{ref}}(x, y, k) \rangle\). After accounting for spectral response of the system, we performed inverse Fourier transform to obtain drOPD map at a set of predefined depth locations based on Eq. (1):

\[
\delta p(x, y, z_{op})_\text{sample} = \frac{k}{2\pi} \arctan(Im(F^{-1}(I_{\text{final}}))/Re(F^{-1}(I_{\text{final}}))),
\]

where \(I_{\text{final}} = \frac{I_{\text{sample}}(x, y, k) - I_{\text{ref}}(x, y, k)}{I_{\text{ref}}(x, y, k)}\). As the tissue thickness varies about ± 0.5 μm for an FFPE tissue section (16), we chose the depth range of 1.35 to 3.15 μm for nanoNAM to be independent.
of thickness variation. The step size of 0.045 \text{ m}, although much smaller than the width of the coherence gate, can be used to capture the gradual change of the refractive index profile along the axial direction (Supplementary Movie S1). We also performed background baseline correction by calculating the phase map on a second background area without any sample present to obtain $\phi_{bg}(x, y, z_{opt})$ by replacing $I_{sample}(x, y, \lambda)$ in Eq. (1) with $I_{bg}(x, y, \lambda)$, so drOPD map is calculated at each depth: $\phi_{OPT}(x, y, z_{opt}) = \phi_{sample}(x, y, z_{opt}) - \phi_{bg}(x, y, z_{opt})$, as shown in Supplementary Fig. S9. Finally, we perform 2D (x-y plane) phase unwrapping based on Goldstein algorithm (17) at each optical depth.

**Colitis patient identification**

Our clinical study was approved by the Institutional Review Board at University of Pittsburgh. We retrospectively reviewed pathology reports from 1995 to 2013 in TIES (a web-based application to search through pathology reports of all UPMC affiliated hospitals) for colitis patients who underwent standard surveillance colonoscopy. High-risk patients are defined as those who subsequently developed HGD or colorectal adenocarcinoma during the follow-up after more than 1 year of the initial surveillance colonoscopy, and low-risk patients are defined as those who did not develop any HGD or adenocarcinoma in the follow-up. We retrieved their FFPE tissue blocks of the colon tissue biopsies in which the pathologic status had no HGD or adenocarcinoma (Supplementary Tables S1 and S2). The slides were reviewed by an expert gastrointestinal pathologist to confirm those cell nuclei for nanoNAM to be histologically normal and grade the inflammation. The person who performed data acquisition and nuclei segmentation was blinded to clinical diagnosis.

**Statistical analysis**

We quantified the nuclear architecture characteristics using two statistical parameters: mean-drOPD and entropy. The mean-drOPD of each nucleus is calculated by taking the average value of all the positive drOPD values in the nuclear architecture map of each nucleus, as the positive drOPD represents increasing change.
of refractive index nuclear architecture. Entropy is calculated for each nucleus using the relation:

$$E_{OPD} = -\sum_{i=0}^{N-1} p(d\rho_i) \log_2 p(d\rho_i),$$

where d\(\rho\) is considered to be a discrete random variable \(d\rho_i\), ranging from \(d\rho_0\) to \(d\rho_{N-1}\), and with probability mass given by \(p(d\rho_i)\) (18). To obtain the scatter plot, we take the average value of mean-d\(\rho\)OPD and entropy of approximately 300 to 500 cell nuclei from a specific pathology type [normal, low-grade dysplasia (LGD), or HGD] for each subject. The LIFETEST procedure (SAS statistical software) was used to compute Kaplan–Meier estimates of the survivor functions and compare survival curves between groups of patients using the log-rank test. The cutoff point for mean-d\(\rho\)OPD and entropy was mathematically determined using an established method (19).

**Calculation of quantitative microscale image features of nuclear architecture**

For a side-by-side comparison between nanoNAM, gold standard of conventional pathology, and digital image analysis, we used standard bright-field images (NA = 0.4, magnification = 45x).

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**Figure 2.**
The nanoNAM on colonic epithelial cells from unstained tissue at different stages of cancer development in an animal model of colitis-associated colon carcinogenesis in which dysplasia is developed in the background of chronic inflammation. The left plot shows the bright-field images of H&E-stained colon tissue, and the right plot shows the corresponding nanoscale nuclear architecture maps of unstained tissue in which the depth-resolved map is averaged along the optical-depth ranges of 1.3 μm to 3.2 μm. The images are from normal cells in control mice (DSS-treated mice, chronic inflammation only; A and B), histologically normal-appearing cells in an AOM/DSS-treated mouse (C and D), histologically low-grade dysplasia (E and F) and high-grade dysplasia in AOM/DSS-treated mice (G and H). Figures A–H and Aii–Hii are the progressively higher zooms of the regions inside the yellow and red boxes. The color bar shows the d\(\rho\)OPD value in nanometers.
of H&E-stained tissue of colon biopsies obtained at the initial surveillance colonoscopy of colitis patients and extracted 12 nuclear features belonging to three broad categories—morphologic, statistical, and textural—typically considered in conventional image analysis–based histopathologic quantification, using the same set of cell nuclei for nanoNAM. The morphologic features describe the area, perimeter, roundness, and elongation of the nuclei. The statistical features characterize the mean, SD, skewness, and kurtosis of nuclear intensity distribution. In addition to looking at the moments of the intensity distribution, we used Haralick features to describe the spatial arrangement of nuclear intensity, thereby characterizing nuclear texture. We specifically looked at four Haralick features: nuclear contrast, pixel correlation, uniformity, and homogeneity (18). The average value for the same set of nuclei used for nanoNAM was used for each patient in the scatter plot. Statistical comparison between low risk and high risk was performed using the two-sided Student t test at 95% confidence interval.

**Results**

**nanoNAM during cancer development using an animal model of colon carcinogenesis**

We first performed nanoNAM to analyze epithelial cell nuclei during cancer progression using a well-established animal model of colorectal carcinogenesis—a chemical-induced colitis-associated colorectal cancer developed in the background of chronic inflammation, in which mice treated with the inflammatory agent dextran sulfate sodium (DSS) and a carcinogen azoxymethane (AOM; Supplementary Methods and Supplementary Fig. S11). We performed nanoNAM on four groups of cell nuclei correlating with cancer progression: (A–B) histologically normal colonic epithelial cell nuclei in DSS-treated mice that developed chronic inflammation without dysplasia, (C–D) histologically normal epithelial cell nuclei in AOM/DSS-treated mice that developed dysplasia, (E–F) dysplastic cell nuclei from LGD and (G–H) HGD, all confirmed by the expert pathologist. We summarized the depth-resolved nuclear architecture maps through an “average” map in which the depth-resolved maps along the optical-depth range of 1.3 to 3.2 μm were averaged, as shown in Fig. 2. The average nuclear architecture maps exhibit progressively higher drOPD, or denser architectural alteration (i.e., increasing change of refractive index), as indicated by more prevalent areas of deeper red color, correlating with cancer progression. Such change is detectable even at an early stage of cancer development when the epithelial cells still appear histologically normal (Fig. 2C and D) than those from mice with just chronic inflammation (Fig. 2A and B). The denser change in nuclear architecture (deeper red color) becomes more prominent and spatially extended in histologically dysplastic cells identified by the expert pathologist, consistent with the well-known nuclear architecture characteristics in cancer cells—coarse aggregation of condensed chromatin—commonly used for cancer diagnosis (7, 8). The HGD nuclei show a higher drOPD value than those from LGD

The image features of nuclear architecture maps are further quantified by two parameters for each nucleus—mean positive drOPD within each nucleus, referred to as “mean-drOPD,” characterizing the degree of denser change of nuclear architecture, and intranuclear entropy characterizing the nuclear architecture disorder. Figure 3A and B show the histogram of mean-drOPD and entropy for the four groups of cell nuclei discussed above. The histogram of mean-drOPD has a wide spread, due to the intrinsic biologic heterogeneity. Overall, however, the histogram of mean-drOPD and entropy show a progressively higher value correlating with cancer progression, from normal nuclei with chronic inflammation, to histologically normal-appearing nuclei from AOM/DSS-treated mice with dysplasia, to LGD nuclei, to the most pathologically advanced HGD nuclei. This result, based on the statistical analysis of several thousand nuclei, further confirms a
progressively more dense and disorganized alteration in nuclear architecture during cancer progression, even at histologically normal stage prior to the detection of dysplasia. Interestingly, the histogram of histologically normal cells from mice that developed HGD shifts toward higher mean-drOPD and entropy, compared with those mice that only developed LGD (Fig. 3Ai–Bi), suggesting the potential of nanoNAM on histologically normal cells to detect the clinically significant HGD. Furthermore, on an individual subject’s level, the average value of single-nucleus mean-drOPD and entropy from all nuclei (~300–600 nuclei per subject) with a specific pathologic type (normal, LGD, HGD) for each subject is shown as a scatter plot in Fig. 3C. The nanoNAM-derived markers from dysplastic cells with pathologically detectable microscopic nuclear abnormality (LGD and HGD) are well separated from normal cells by a large difference. Importantly, the nanoNAM of cells that appear histologically normal to the expert pathologist can also distinguish those mice with just chronic inflammation (diamond) from those mice that developed dysplasia (square), even though with a smaller difference than the distinction between normal (circle and square marker) and dysplastic cells (triangle and diamond marker). This result shows the promise of nanoNAM to detect early-stage cancer development. We further confirmed that nanoNAM-derived progressively denser alteration of nuclear architecture is indeed characteristic of cancer progression, rather than the side effect of the drug treatment or animal specific, with another animal model of a spontaneous development of colorectal cancer – Apcmin/+ mice that develop multiple intestinal neoplasms (Min) due to a germ-line mutation in adenomatous polyposis coli (Apc) gene (Supplementary Methods and Supplementary Fig. S12).

nanoNAM to predict “future” cancer progression in ulcerative colitis patients

As a proof-of-concept, we demonstrate the potential clinical use of nanoNAM to predict “future” cancer progression in a retrospective clinical study with ulcerative colitis patients who had up to 13 years of surveillance colonoscopy follow-up. Using Pathology Database at University of Pittsburgh Medical Center, we identified 15 high-risk patients (or progressors) who developed HGD or colorectal cancer after at least more than 1 year. These high-risk patients are matched with 18 low-risk patients (or nonprogressors) who did not develop any HGD or colorectal cancer during the follow-up, in which none of the available clinical and pathologic factors has any bias between low-risk and high-risk group (Supplementary Table S1). To ensure that the low-risk patients indeed did not develop cancer, we selected those patients with an average of 8.7 follow-up years, compared with an average of 4.3 follow-up years in high-risk patients. The archived FFPE tissue block of colon tissue biopsies obtained at the time of the initial surveillance colonoscopy was retrieved and nanoNAM was performed on the histologically normal epithelial nuclei on the unstained slide from the initial colon tissue biopsies. As most patients had no dysplasia with a small percentage of tubular adenoma, indefinite for dysplasia and LGD that represent the actual clinical scenario, nanoNAM focused on those histologically normal cells to ensure the consistency of pathology type, confirmed by an expert gastrointestinal pathologist (D.J. Hartman).

Figure 4 shows the representative drOPD maps (averaged along the optical-depth range of 1.3 to 3.2 μm) of the initial normal tissue biopsy from a low-risk ulcerative colitis patient who did not develop HGD or colorectal cancer during 7 years of follow-up, and a high-risk ulcerative colitis patient who developed colorectal cancer after 7 years. The cell nuclei from the high-risk patient show denser architecture alteration, compared with those from the low-risk patient, consistent with our observation in the animal models of colorectal cancer progression.

We then calculated the mean-drOPD and entropy of each nucleus for approximately 300 to 600 normal-appearing nuclei per patient and the histogram of single-nucleus mean-drOPD and entropy for each patient. As shown in Fig. 5, the histogram of high-risk patients shifts toward higher mean-drOPD with a broader distribution (solid lines), suggesting denser alteration of nuclear architecture and greater nuclear heterogeneity, compared with those from low-risk patients (dashed lines). The mean-drOPD and entropy values are then averaged over all nuclei for every patient to generate single-patient mean-drOPD and entropy, used as the patient characteristics. As shown in the scatter plot (Fig. 6A), the nanoNAM-derived single-patient mean-drOPD and entropy separate high-risk from low-risk group and correctly classify 12 of 15 high-risk and 15 of 18 low-risk patients. Figure 6B shows the Kaplan–Meier curve for time-dependent cancer progression using entropy as the estimator. Intranuclear entropy shows a high level of statistical significance in predicting HGD/ colorectal cancer progression over time (log-rank test, P value =...
0.000045). The similar statistical significance is observed using mean-drOPD as the estimator (P value = 0.0033).

Further, we compared our results with both gold standard and widely used quantitative analysis of standard histology images. First, our expert pathologist (D.J. Hartman) went back and reviewed all the slides used for nanoNAM and did not identify any distinct pathologic features in high-risk patients (Supplementary Table S2). Next, we evaluated whether quantitative micro-scale nuclear architecture from bright-field image of H&E-stained tissue can distinguish high-risk from low-risk patients. On the same set of cell nuclei analyzed by nanoNAM, we calculated 12 commonly used microscale image features (i.e., 4 morphologic, 4 statistical, and 4 textural), which do not separate high-risk from low-risk patients (Fig. 7) as much as the nanoNAM-derived features (Fig. 6B). The statistical analysis of these 12 microscale features also shows no significance (P value > 0.4; Supplementary Table S3). Therefore, we further confirmed that it is indeed the nanoscale sensitivity of nanoNAM that detects early cancer progression, which cannot be identified by both pathologic evaluation and quantitative image features of microscale nuclear architecture.

**Discussion**

Our nanoNAM is a promising approach to address the highly unmet clinical need of personalized risk assessment for patients...
who are at risk for developing cancer, but not presented with clinically significant lesions. nanoNAM does not resolve nanoscale structure as super-resolution imaging, but measures the intrinsic nuclear density alteration via nanoscale drOPD at a sensitivity of 1 to 2 nm. The technical advance of nanoNAM enables the analysis of clinical FFPE tissue, the most common form of preserved archival clinical sample, without being compromised by the variation introduced by imperfect clinical sample preparation. The low-coherence spectral interferometry built into a standard glass slide and closely matched refractive index between the tissue and mounting medium provides the depth-resolved imaging capability to eliminate the variation in tissue thickness; the robust image registration based on high-contrast transmission quantitative phase image allows reliable identification of cell nuclei without being confounded by nuclear staining variation; the use of low coherent light source and a common-path interferometry configuration minimizes multiple noise sources to ensure the reproducibility of nanoscale sensitivity of 1 to 2 nm at a single-nucleus level, crucial for detecting early changes of nuclear architecture in cancer progression in clinical samples, as demonstrated by our extensive validation in animal models and patient samples. Unlike many biochemistry-based methods, nanoNAM maintains spatial and pathologic context of each cell nucleus, and can serve as an adjunct to pathology. Further, although the cost of nanoNAM will depend on its eventual clinical adoption, a few technical attributes make it potentially low cost. It uses intrinsic scattered light with simple low-NA optics at a clinically feasible throughput (~500 epithelial nuclei in 30 minutes); sample preparation is based on standard low-cost clinical tissue processing (e.g., tissue sectioning, H&E staining) without expensive chemicals.

Although the ability to detect nanoscale changes in cell nucleus has been demonstrated by several other optical imaging techniques, their applicability to assessing cancer progression risk on routine clinical FFPE tissue is limited. Super-resolution fluorescence microscopy (e.g., STORM, PALM, STED; refs. 20–22) offers superb nanoscopic image resolution, but the complex fluorescence staining has a limited performance on clinical FFPE tissue, and their low-throughput and high-cost instrumentation limits their routine clinical use. Other optical techniques such as optical coherence phase microscopy (11–13), transmission quantitative phase microscopy (23), digital holographic microscopy (24), confocal light absorption and scattering microscopy (25), and partial-wave spectroscopy (26) can detect structural changes at nanoscale sensitivity using scattered light, but their ability to assess nanoscale nuclear architecture on clinical FFPE tissue can be confounded by either tissue thickness variation, dependence on over-simplified models to interpret optical signals, or lack specificity to cell nuclei; adding nuclear stains affects measured optical signals.

To demonstrate the ability of nanoNAM in detecting early cancer progression, we conducted extensive validation using animal models of colon carcinogenesis and human colitis patients at risk for developing colorectal HGD or adenocarcinoma. By analyzing both histologically normal-appearing cells and pathologically dysplastic cells, we identified gradually denser change of nuclear architecture that correlates with cancer progression, in which the early-stage cancer progression in histologically normal cells prior to dysplasia can be detected by nanoNAM. We demonstrated the clinical utility of nanoNAM to predict ‘future’ colon cancer progression in ulcerative colitis patients with well-documented clinical outcome, by nanoNAM of biopsies obtained at the initial surveillance colonoscopy well before patients developed HGD or adenocarcinoma. The nanoNAM-derived markers, mean positive drOPD (representing denser nuclear architecture alteration) and entropy (representing intranuclear heterogeneity), predict risk of progression with an overall accuracy of 85% (12 of 15 high-risk and 15 of 18 low-risk patients). In comparison, the gold standard (conventional pathology) and digital image analysis using the same set of cell nuclei from each patient did not distinguish high-risk from low-risk patients with statistical significance.

Both pathologic and molecular evidence support our finding that cells undergoing neoplastic transformation accumulate progressive abnormality in nuclear architecture and density distribution that eventually manifest as pathologically detectable characteristics of cancer cells. At the pathologic level, it is well known that nuclei of cancer cells differ in the amount and distribution of condensed heterochromatin from their normal progenitor cells, which is widely used for cancer diagnosis and prognosis (27). At the molecular level, recent studies showed that nuclear architecture with respect to the distribution of condensed and open chromatin regions directly affects mutation rate variation (28) and widespread DNA methylation (29, 30). A recent electron microscopy study reported an increase in the condensed heterochromatin content and clump size in preneoplastic cell nuclei that...
appear histologically normal (31), which also suggests the increased density of nuclear architecture in the early-stage cancer development.

Conventional pathology is the gold standard and still remains the first-line diagnostic tool. The nanoNAM can complement conventional pathology as a cost-effective clinical test to risk-stratify patients for developing cancer, when the cancer progression risk of the patients is unknown. Although this technique is demonstrated in colon carcinogenesis, given that the alteration in nuclear architecture is one of the most universal characteristics in many types of cancer, it may be applicable to other tumor types. As cancer generally develops over a long period of time, the ability to analyze PFFE tissue via the retrospective study allows us to perform our proof-of-concept in patients with accurate clinical phenotype and well-matched low-risk and high-risk group that would otherwise take a decade in a prospective setting. Because progression from ulcerative colitis to adenocarcinoma is very rare, there are only a limited number of high-risk patients that can be identified in a single large medical center. Despite our promising results, future widespread clinical use will, therefore, require a large-scale multicenter clinical study for additional validation, and the technical issues related to effect of different fixation from multiple institutions have to be addressed. Nevertheless, we demonstrate that nanoNAM has a great potential as a cost-effective routine clinical test to predict each at-risk patient's progression risk and personalize their surveillance and treatment strategies to eventually reduce overtreatment and improve early detection of malignancy.

Figure 7. Scatter plots of 12 quantitative image features (nuclei area, perimeter, roundness, elongation, mean, SD, skewness, kurtosis, contrast, homogeneity, correlation, and uniformity) of microscale nuclear architecture extracted from the microscopic bright-field images of H&E-stained tissue of colon biopsies obtained at the initial surveillance colonoscopy for low-risk (solid dots) and high-risk (hollow circles) colitis patients. Each scatter plot shows two of the 12 image features.
Disclosure of Potential Conflicts of Interest

S. Uttam and H.V. Pham have ownership interest (including patents) in patent application US20150204728A1 and is a consultant/advisory board member for Nanovision Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.V. Pham, J. LaFace, B. Leibowitz, J. Yu, D.J. Hartman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Uttam, H.V. Pham, D.J. Hartman, Y. Liu
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