Supplementary Methods

***Mathematical expression of spectral interference signals for drOPD mapping***

For a spatially incoherent source, the detected backscattered wave from the sample is superimposed with the reference wave reflected at the glass slide-sample interface, resulting in an interference signal:

 (1)

where *S*(*k*) is the power spectrum of the source, is the reflection coefficient of the reference wave, is the scattering coefficient of the sample at depth *z*, *Z* is the total sample thickness and *n*(*z*) is the refractive index profile along the axial *z*-direction. The Fourier inverse of Eq. (1) results in Eq. (2): (2)

with and. Equation (2) is a convolution of the source correlation function Γ with the interference signal, showing how the source correlation function serves as an implicit coherent window that localizes the information at each optical depth to be within the coherence length. The phase of the Fourier transformed signal at any pre-defined *z*opl captures nanoscale alterations in OPL at the depth *z*opl, given by Eq. (3): (3) where is the center wavelength, is the depth-resolved optical path length difference (drOPD) at a specific optical depth location , and the phase term is determined by Eq. (4): (4) (Im and Re denote the imaginary and real parts of the complex convolution). The drOPD is not limited by the axial resolution of optical system, but limited by the system noise.

 Equation (4) describes the phase measurement made at *zopl* and its physical interpretation given by Eq. (5): (5), where , the actual reflection profile of the scattering object, is the gradient of the object’s reflection profile and is the source correlation function determined by the spectral bandwidth that defines the width of coherent gate, which also determines the axial resolution of the optical system (2µm in our system). This equation shows that the depth-resolved phase term captures the combined effect of two aspects of refractive index profile of the object within the coherence gate. The first term of Eq. (5), given by , is the sub-resolution offset that measures the sub-resolution deviation between the optical depth where the phase measurement is made, and the optical depth corresponding to the weighted-center of the coherence-gated refractive index profile of the object around the optical depth being probed. This deviation exists because the weighted-center, due to alteration in the refractive index profile of the object within the coherent gate centered at, can be different from. allows the estimation of this difference. The second term of Eq. (5), given by the fraction, is an estimate of the average rate-of-change of the refractive index profile (or mean spatial-frequency) within the coherence gate at the optical depth being probed. This can be understood by observing that the imaginary part in the numerator – integration (written as a convolution, , with ) of the baseband representation (indicated by the superscript *Kc*, the center frequency of the source) of within the coherence gate – is a measure of the mean gradient of refractive index profile within the coherence gate, while the real part of the denominator – integration of the baseband representation of within the coherence gate – measures the net change in the refractive index profile within the coherence gate. Together, their ratio estimates the average rate-of-change, or mean spatial-frequency, of the coherence-gated refractive index profile. We note that the imaginary and real parts of the numerator and denominator respectively make the representation consistent with the arctangent for phase representation. When the coherent gate is moved along the axial direction with a step size much smaller than the width of the coherence gate (0.045 µm in our case), the drOPD captures the gradual change of the refractive index profile along the axial direction. When drOPD is averaged over a certain optical-depth range, the effect of sub-resolution offset,, the first term of Eq. (5), is cancelled out such that the average drOPD primarily captures the effect of the rate-of-change (mean spatial-frequency) of the coherence-gated refractive index profile. Therefore, the drOPD quantifies the changing (increasing or decreasing) optical density within the gate, and its mean heterogeneity.

***Numerical simulation to illustrate depth-resolved drOPD imaging***

Figure S1 illustrates the concept of depth-resolved imaging with numerical simulation. To perform the numerical simulation, we consider a low-coherence spectral source with same spectral bandwidth as our experimental light source. A Gaussian random process based refractive index profile is generated with the step size of 1nm (Fig. S1A). A wave transfer matrix is used to implement wave propagation and reflection through the refractive index profile. For each wavelength, the back-reflected waves from all depths are interfered with a reference wave to generate the spectral interference signal. The self-interference of the reference and back-reflected sample waves are ignored. After normalizing by the shape of the source spectrum, the Fourier transform of the spectral interference is performed and its phase as a function of optical depth is computed using Eq. (1). The phase is unwrapped and the phase ramp due to the center frequency (corresponding to the center wavelength) of the light source is subtracted from it. The gradient of the resulting phase is calculated. To get at a particular optical depth of interest, the phase gradient is integrated within the coherence gating around that optical depth. Finally is computed by multiplying by . As shown in Fig. S1A, the structure of the scattering object is described by the refractive index profile along the optical depth, which does not have any strong interface, mimicking the internal architecture of cell nucleus. A local structural change is represented by a small increase of refractive index (or increased rate-of-change) at Location 1 and the corresponding decreased refractive index change at Location 2, as shown in the figure insets of Fig. S1A. The phase profile of Fourier transform of spectral interference signal captures the local sub-resolution change of refractive index at their respective optical depth centered around the coherent gate, as shown in Fig. S1B. Increasing local refractive index (or rate-of-change of refractive index) leads to positive drOPD. It should be noted that such internal structural changes cannot be detected by conventional transmission phase imaging, as the accumulative phase along the entire sample depth remains constant. The drOPD value goes up with increasing refractive index (Fig. S2). This result shows the ability of drOPD to detect minute local change in refractive index profile at a specific depth.

***Depth-resolved imaging via drOPD***

To experimentally confirm the depth-resolved imaging capability, we used an unstained slide from a thin section (5 µm) of a cell block containing HeLa cells embedded in a polymer network of HistoGel®. Figure S3A shows the bright-field image of cell section which shows cells embedded in polymer network. Its quantitative phase map obtained from transmission diffraction phase microscope (1) is shown in Fig. S3B, in which the accumulative phase (or optical path length (OPL)) for light passing through the entire sample is measured. Figures S3C-E show the corresponding drOPD maps at three optical depths (*z*opl = 1, 2, and 5 µm). At a superficial optical depth of 1 µm (Fig. S3C), the drOPD map shows both cells and polymer network. At an optical depth of 2 µm (Fig. S3D), the polymer network is barely visible, indicating the depth sectioning capability of drOPD mapping. At a deeper depth of 5 µm (Fig. S3E), the polymer network becomes invisible. The cell circled in red is visible at 1 and 2 µm, but disappears at 5 µm; while the cell circled in black can be seen in all three depths, indicating the thickness variation of different cells (thicker cell circled in black than the one circled in red). This thickness difference is further supported by transmission quantitative phase map (Fig. S3B), in which the cell circled in black shows a higher optical pathlength (OPL) than the one circled in red. Although both transmission phase map and drOPD map measure phase changes, this result highlights the complementary nature of these two approaches: drOPD map can detect internal cell structural characteristics independent of cell thickness, but has a low image contrast; while transmission phase map measures the integrated OPL along the entire sample depth that is sensitive to thickness, but provides a high image contrast. In both methods, the unstained image does not provide sufficient contrast to unambiguously identify cell nuclei, suggesting the need for a different contrast mechanism.

***Sample preparation for nanoNAM***

We first prepared an unstained slide from a recut from FFPE tissue block, sectioned at 5 µm thickness using a microtome, which is placed on the coated glass slide as described above. Then the tissue is deparaffinized, rehydrated in ethanol series with graded alcohol (100%, 95%, 70% and 50%) and then dehydrated back to xylene in the reverse order. The unstained slide is then coverslipped with mounting medium (micromount®, n = 1.50 for dried mounting medium, Surgipath, Leica). After drOPD mapping and transmission phase imaging on the unstained tissue, the slide is immersed in xylene overnight to remove the coverslip. The de-coverslipped tissue slide is then rehydrated in ethanol series with graded alcohol (the same as the processing for unstained slide), stained with hematoxylin and eosin, and then dehydrated back to xylene in reverse order of grade alcohol. The slide is then coverslipped with the same mounting medium. The H&E-stained slide was imaged with bright-field and transmission phase microscopy for pathology correlation, nuclei segmentation and image registration.

***nanoNAM instrument setup***

The detailed schematic of nanoNAM is shown in Fig. 1A2 (main text). A low-coherence white light Xenon lamp (EQ-99, Energetiq) is used and the collimated beam passes through a high-speed acousto-optical tunable filter (AOTF) to tune the wavelength (480-700 nm) at a spectral resolution of 1-3 nm, shared by three imaging modules. A standard microscope frame (AxioObserver, Carl Zeiss) is used. A flipping mirror (RM) is used to direct the illumination into different modules. When RM is at “off” position, the illumination beam is reflected by beam splitter (BS), and focused onto the back focal plane of the objective (OBJ) by achromatic lens L1 to achieve a uniform field of view (~250μm diameter). The reflected reference wave and backscattered light from the sample are collected with OBJ, achromatic lens (L2) and projected onto the camera sCMOS1 (pco.edge, PCO-TECH). To avoid double transmission (i.e., the reflected light at coverslip/air interface passing through the sample), we used a right-angle prism (Edmund Optics) on top of the coverslip with immersion oil (n = 1.515) in between to deflect the light outside the microscope system. The images at ~230 wavelengths tuned by AOTF are recorded with a total of 20 seconds, repeated 4 times to average out the noise. We removed the inter-user variation in identifying focal plane via an automatic focusing method (see below and Fig. S5), with the objective mounted on an objective nano-positioner (Edmund Optics). We also corrected for wavelength-dependent focal plane shift and image distortion (see below and Figs. S6-S7). Then RM is placed at “on” position, the beam is used for trans-illumination (blue dashed line in Fig. 1A2 of main text) for transmission phase imaging based on the configuration of diffraction phase microscope (1). At the image plane located at the side port of the microscope frame, a transmission grating G (Edmund Optics) is used to create different copies of the emerging field. At the back focal plane of the lens L3, the DC component filtered from the 0th-diffraction order and the entire 1st-order diffracted beam are collected and interfere on the camera sCMOS2 at a wavelength of ~560 nm. After the unstained tissue is imaged, the slide is stained with H&E, which is then imaged with bright-field (collected by sCMOS1) and transmission phase modules. The slide is mounted on a motorized high-precision translational stage (MLS203-2, Thorlabs) to record the same sets of locations before and after staining and for automatic lateral scanning of multiple areas.

***Automatic focusing***

To ensure the consistency of focal plane position by different users, we used a simple derivative-based algorithm to perform autofocusing. At each image location, a fixed wavelength is chosen, and z-stack reflectance images are acquired. The squared gradient algorithm (2) is then used to define the position of the focal plane. Specifically, at each image, the focus measure is calculated as , where  and *θ* is the gradient threshold. Then, the focus plane is found by . Figure S5 shows the focus measure *F*(*z*) as a function of focal plane position (z), and the focal plane is defined as the axial z position with the maximum value of *F*(*z*).

***Correction for wavelength-dependent focal plane shift***

Despite the achromatic optics are used throughout the entire optical system, the chromatic aberration is still present in the optical system with a small focal shift of several microns in the spectral range of 490-680 nm. In order to further correct for this chromatic aberration, we developed a chromatic correction curve. Specifically, we used 18 wavelengths (evenly spaced in the spectral range of 490 to 680 nm, and for each wavelength, the above autofocusing algorithm is used to obtain the focal shift curve along the entire spectral range. Figure S6 shows the average focal shift curve with error bar indicating the standard deviation. To minimize the effect of noise, we fit this curve with a fourth order polynomial (shown in the blue curve) to find the focal-plane position at each wavelength.

***Chromatic correction***

Due to chromatic aberration, there is a wavelength-dependent image distortion. To correct for such effect, a region-of-interest on the imaging standard (Figure S7a, Image analysis micrometer, Edmund Optics) was imaged over the spectral range of interest (490 to 680 nm) to obtain point estimates of location-dependent spectral drift at nine locations, corresponding to the centroids of a nine-square grid covering the entire field-of-view. The point estimates were used to estimate the distortion field over the field-of-view for the spectral range using spline-based interpolation. The distortion field for a specific wavelength is the estimate of the location-dependent translation that the image undergoes with respect to the image at the first wavelength. Figure S7b illustrates this distortion as a difference image between the two wavelengths. Distortion correction reverses this translation to be within 2-5 pixels or ~500 nm, which is below the image resolution, as shown in the difference image in Fig. S7c. By extending this correction to the entire spectral range, we obtained the undistorted spectral data cube, which is applied to each measured spectral cube before the phase map calculation.

***Image registration* *of unstained and stained tissue***

Co-registration between unstained and stained tissue images is necessary in identifying nuclei locations in the unstained tissue image from segmented nuclei locations in the stained tissue image. A robust image registration requires similar image features present in the two images to be registered, but the dramatically different image features in the low-contrast drOPD image of unstained tissue and the stain-absorption-based contrast of bright-field image of H&E-stained tissue present a real challenge. We observed that phase contrast generated by transmission phase is less affected by staining, as shown in Fig. S8(A-B), where transmission phase images of unstained and stained tissue have similar contrast profiles. We exploit this similarity in phase contrast to perform image registration. Specifically, using Otsu’s method *(26)*, we individually threshold both images to identify the foreground landmark features in each, which are nearly identical in unstained and stained phase images. Normalized cross-correlation between the segmented landmark features in the unstained and stained images are then used to estimate the translation, and log-polar fast Fourier transform (3) is used to estimate the scaling and rotation. With our microscope slide holder, we observed that translation correction was sufficient for robust registration, because focus-correction eschewed the need to correct for scaling, and the slide holder successfully minimized rotations. Figures S8(C-D) show the overlaid transmission phase images of unstained and stained tissue before and after image registration, confirming the proper co-registration of the two images. The registration was performed under the assumption that the mounted tissue sample did not move relative to the slide during tissue staining process. To ensure the validity of this assumption, we optimized tissue processing protocol with two strategies: (1) The glass slide is coated with poly-L-lysine to ensure good adherence of the tissue onto the slide; (2) The stepwise hydration-dehydration using graded alcohol (a step to remove paraffin) is used to in both unstained and stained tissue processing to ensure minimal shrinkage. This protocol has been extensively tested to work in ~99% of the cases, with no tissue position shift after staining.

***Cell nuclei segmentation***

A segmentation GUI was developed to (1) provide the operator with the ability to perform both manual and semi-automatic segmentation. The latter was based on constrained region-growing where the seed location within the nucleus was identified by the operator. The constraint was a relaxed convexity constraint to prevent unstable region growing and therefore ensuring a well-defined nuclear boundary. (2) It was also used to streamline the association between the segmented nuclei and their pathological class. Only the epithelial cell nuclei are segmented and confirmed by a pathologist. If the boundary of the single nucleus cannot be clearly determined either by direct visualization or semi-automatic segmentation method, that nucleus is not selected for analysis.

***Reproducibility of drOPD***

We have conducted extensive test to characterize the analytical sensitivity and reproducibility of the optical system. First, we characterized phase stability as a key characteristic as it determines the smallest detectable structural changes. The temporal phase stability is characterized by taking the continuous measurement on an unstained tissue section slide for ~3 hours. The structural sensitivity (drOPD value) quantified by the average of pairwise difference of different phase maps and the drOPD sensitivity over 60 by 60 pixel area (~ size of a single nucleus) is 0.9 nm. Second, we measured the same unstained tissue section once a day at three different acquisition time (20ms, 50ms and 100ms) over one-week period, and quantified the reproducibility of the measured nanoscale nuclear architecture for the same segmented nuclei. Different acquisition time is used to mimic the possible intensity variation. As shown in Fig. S10, the box plot shows the variation of the mean-drOPD of each single-nucleus for the same set of nuclei on the tissue section over one-week period. The pairwise difference between different acquisition time and different days is centered around 1 nm (indicated by the red line) with an upper bound of ~2 nm.

***Cell block processing***

Cell block used in Fig. S3 was processed in the Tissue and Research Pathology Service at University of Pittsburgh. In brief, cells fixed in 4% formalin were first concentrated by spinning cells in a centrifuge tube until a cell pellet was formed. Then the HistoGel (Thermo Scientific) was added to the cell pellet. After the histogel embedded with cell pellet solidifies, 4% formalin was added to remove the cell block (gel button with specimen cells) from the container. The cell pellet was then embedded in embedded in paraffin, as a paraffin-embedded cell block. Each slide from the cell block was prepared following the same standard tissue histology processing protocol except no staining used: the paraffin block was sectioned at 5µm thickness; and a cell section was placed on the glass slide, then paraffin was removed, and coverslipped with the mounting medium (micromount®, n = 1.50, Surgipath, Leica).

***Animal models***

All animal studies were performed in accordance with the institutional Animal Care and Use Committee of the University of Pittsburgh. All mice were housed in micro isolator cages in a room illuminated from 7:00 AM to 7:00 PM (12:12-hr light-dark cycle), with access to water and chow *ad libitum*.

*Colitis-associated mouse model of colorectal carcinogenesis*

To induce colitis as a control for chronic inflammation, three C57BL/6J mice (JAX stock# [000664)](http://jaxmice.jax.org/strain/000664.html) at 5-6 weeks were treated with three 7-day cycles of the inflammatory agent dextran sulfate sodium (DSS) at 2.5% in the drinking water. To induce colitis-associated colorectal cancer (CRC), three C57BL/6J mice were first injected with 12.5 mg/kg azoxymethane (AOM), then treated with three 7-day cycles of 2.5% DSS, as shown in Fig. S11 (4). After 2 months of recovery, the mice were sacrificed and the colon tissue was harvested and prepared in in bundles of 0.5 cm segments (5). As confirmed by the expert pathologist, three DSS-treated mice developed chronic inflammation without any dysplasia; two DSS/AOM-treated mice developed low-grade dysplasia (LGD) and one mouse developed high-grade dysplasia (HGD).

*Apc*Min/+ *mouse model*

A total of 12 female mice were included in this study and fed the AIN-93G diet to facilitate the development of polyps from 4 weeks of age until sacrifice (6). A first set of three C57BL/6J wild-type mice and three age- and sex-matched C57BL/6J and *Apc*Min/+ (JAX stock number 002020) mice were sacrificed at 6 weeks of age. A second set of three *APC*+/+ mice and *Apc*Min/+ mice were sacrificed at 12 weeks of age. The 6-week *Apc*Min/+ mice did not show any visible tumor or dysplasia as confirmed by the pathologist; whereas those 12-week *Apc*Min/+ mice had developed multiple visible adenomatous polyps and histologically visible dysplasia in their small intestine. The small intestines tissue was removed, washed with phosphate buffered saline, and prepared in bundles of 1 cm segments as described. We fixed the tissue in 10% neutral buffered formalin for over 24 hours, and embedded the tissue in paraffin block. A segment of colon (both proximal and distal parts) was cut, and the tissue was placed in 10% neutral buffered saline for over 24 hours. Then the tissue was embedded in paraffin block.

To prepare an unstained slide, we obtained 5-µm tissue section, placed on the coated slide with the same protocol as described in *Materials and Methods*. We performed nanoNAM on three groups of cell nuclei: (1) histologically normal intestinal epithelial cell nuclei from 6-week wild-type and age-matched *Apc*Min/+ mice, an early-stage of carcinogenesis without visible tumor; (2) histologically normal intestinal epithelial cell nuclei at 12 weeks; (3) histologically dysplastic cells at 12 weeks, a cancer stage with visible tumors in the small intestines.

As shown in Fig. S12, at 6 weeks, it is an early-stage cancer development, with no visible tumor or dysplastic tissue found in the small intestine; while at 12 weeks, visible tumors can be seen in the small intestinal tissue of *Apc*Min mice. In the histologically normal intestinal epithelial nuclei, there are progressively higher mean-drOPD and entropy values in 6-week and 12-week *Apc*Min/+ mice compared to those normal cell nuclei from the age-matched wild-type mice; in histologically dysplastic cell nuclei, higher prevalence of higher mean-drOPD or deeper red in the nanoscale nuclear architecture maps can be seen compared to those of normal cells. This result confirmed our previous finding in AOM/DSS-treated mouse model, 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.

**Supplementary References:**

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