Calculating Disorder strength using PWS microscopy:

Theoretically, the disorder strength ($L_d$), is defined as: $L_d = \delta n^\alpha \times l_c^\beta$ where $\delta n$ is the standard deviation of the refractive-index (mass-density) variations and $l_c$ is the correlation length of these variations where, the value of $\alpha$ is in the range of 1 to 2, depending on the amplitude of intracellular refractive index fluctuations relative to the refractive index mismatch between a surrounding medium and a cell (for a fixed cell situated on a glass slide, $\alpha=1$), while $\beta$ depends on the numerical aperture of the system and can be approximated as 1. The partial wave spectroscopic (PWS) microscopy instrument attempts to quantify this parameter from a given specimen. PWS instrument used for collecting data in this study (1) generates a three dimensional data cube for the reflected intensity $I(\lambda;x,y)$ where $(x,y)$ refers to a specific pixel in the object plane and $\lambda$ is the wavelength. After normalizing each pixel by the corresponding incident light profile (using a mirror), we generate $R(\lambda;x,y)$ which is referred to as the fluctuating part of the reflection coefficient. The spectral fluctuations between wavelength range of 550 - 700 nm (to avoid the lower intensity regions and prominent absorption peak of the staining reagents at the lower wavelengths and also to get rid of the noise from the camera) are further analyzed by means of 1D mesoscopic light transport theory (2-5) to obtain $L_d$:

$$L_d = B \frac{n_0^2}{2k^2} \langle R \rangle \left[ \frac{\Delta k^2}{-\ln(C(\Delta k))} \right]$$

(1)

where $n_0$ is the background refractive index, $k$ is a wave-number and $\langle R \rangle$ is calculated by taking the root mean square value of the spectrum $R(k)$ while $C(\Delta k)$ is the autocorrelation function of the spectrum $R(k)$.

$$C(\Delta k) = \langle R(k)R(k + \Delta k) \rangle / \langle R(k)R(k) \rangle$$

(2)

Furthermore, $-\frac{\ln(C(\Delta k))}{(\Delta k)^2}$ is obtained by fitting a linear slope to $-\ln(C(\Delta k))$ versus $(\Delta k)^2$.

Thus a map of disorder strength $L_d(x,y)$ is obtained from each pixel $(x,y)$. From the two dimensional map $L_d(x,y)$ of each cell the mean intracellular disorder strength $L_{d(c)}^{c}$ (the average $L_d(x,y)$ over $x$ and $y$) and the standard deviation of intracellular disorder strength values, $\sigma^{(c)}$ is obtained.

Validation of PWS calculated disorder strength ($L_d$):

We have reported earlier various approaches in order to validate PWS-measured disorder strength parameter and its accuracy in measuring a physical quantity of a given sample (6). In particular, we performed 3 studies to validate nanoscale sensitivity of PWS: (i) rigorous computational experiments using finite-difference time-domain (FDTD) simulations (7), (ii) experiments with nano-structured polystyrene bead phantoms, and (iii) experiments with biological cells. These studies are discussed in detail in reference(6).
(i) In brief, our computational experiments were based on FDTD simulations of light propagation in random cell-emulating media. We considered a homogeneous dielectric slab with random refractive index fluctuations around the background refractive index $n_0 \approx 1.38$ (similar to the average refractive-index of a biological cell). Furthermore, the standard deviation, $\delta n$ of the fluctuations was varied from 0.01 to 0.05 and the correlation length $l_c$ was varied from 5 to 45 nm while the thickness of the sample $L$ was varied from 1.5 to 4 $\mu$m. The results of the FDTD-simulations under these initial conditions demonstrated that the values of $L_d$ obtained from the spectral analysis were in good agreement with its true value ($r^2 \approx 0.91$)(6). Moreover, $L_d$ linearly depends on $l_c$ for $kl_c < 1$ and thus, in principle, there is no limitation on the minimum $l_c$ that can be assessed by PWS microscopy.

(ii) In our experiments with deterministic nano-structured bead phantoms, we used aggregated polystyrene nanospheres (refractive index, $n \approx 1.59$ and $\delta n = 0.29$) of known bead-sizes, 20 to 125 nm (i.e., $l_c = 5$ to 40 nm) (8). We found a good agreement ($r^2 \approx 0.97$) between $L_d$ measured by using the PWS instrument and the expected disorder strengths. This experiment also served the purpose of calibrating the system (6).

(iii) We confirmed the application of mesoscopic-theory based analysis for real biological cells (6). It is applicable if the following three conditions are satisfied: (1) $\langle R \rangle \ll 1$, (2) the probability distribution function (p.d.f) of $\langle R \rangle$ is log-normal, (3) $C(\Delta k)$ is a Gaussian function. We would like to note that all these three conditions are satisfied during the analysis of rectal colonocytes investigated in this study.

**Biological Mechanisms underlying disorder strength differences:**
There have been several reports which indicate cytoskeletal and chromatin architectural alterations in colonic cells during carcinogenesis (9-12). All these changes are reported using biological assays however their quantification has not been performed or their translation to clinical practice has been limited. Hence, we developed PWS microscopy to quantify these cellular nanoscale architectural changes in a parameter, disorder strength ($L_d$). Because the refractive index increment is (within the accuracy of measurements) independent of the chemical composition of the macromolecules (with the proportionality coefficient fairly constant $\sim 0.186$ mL/g for various macromolecules (13-14)), $L_d$ quantifies local macromolecular compaction/condensation. That is, Due to the linearity between the local refractive-index and mass-density, i.e. $n = n_o + \chi \rho$ where $n_o$ is the refractive index of the medium, $\rho$ is the portion of intra-cellular solids by volume and $\chi$ is a proportionality coefficient with a constant value of $\sim 0.186$ mL/g), we can derive an equation for $L_d$ based on this relationship for our case using definition of $L_d : L_d = \chi < \Delta \rho > l_c$. The exact molecular origin of this macromolecular condensation depends on the intracellular location where $L_d$ is measured. For example, at a
particular location within the nucleus there is a higher chromatin compaction, hence there is higher mass-density leading to higher $\delta n$ and $l_c$ resulting in higher $L_d$. Thus, in the nucleus, $L_d$ increase is a measure of chromatin condensation.

Recently, we demonstrated that differences in $L_d$ are contributed in part by the cytoskeletal organization present in both, the nucleus and the cytoplasm(1) in the colon cancer cell-lines. Moreover, our recent simulation work based on Brownian dynamics and electron-microscopy studies suggested that $L_d$ increase in the nucleus parallels chromatin compaction (15-17). Thus, $L_d$ measured by PWS nanocytology reflects the quantity and organization or spatial arrangement of cellular building blocks (such as cytoskeleton, higher-order chromatin structure) in the colonic cells.

Reference: