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Measuring nanoscale chromatin heterogeneity with partial wave spectroscopic microscopy

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Abstract

Despite extensive research in the area, current understanding of the structural organization of higher-order chromatin topology (between 20-200 nm) is limited due to a lack of proper imaging techniques at these length scales. The organization of chromatin at these scales defines the physical context (nanoenvironment) in which many important biological processes occur. Improving our understanding of the nanoenvironment is crucial because it has been shown to play a critical functional role in the regulation of chemical reactions. Recent progress in Partial Wave Spectroscopic (PWS) Microscopy enables real-time measurement of higher-order chromatin organization within label-free live cells. Specifically, PWS quantifies the nanoscale variations in mass density (heterogeneity) within the cell. These advancements have made it possible to study the functional role of chromatin topology, such as its regulation of the global transcriptional state of the cell and its role in the development of cancer. In this chapter, the importance of studying chromatin topology is explained, the theory and instrumentation of PWS are described, the measurements and analysis processes for PWS are laid out in detail, and common issues, troubleshooting steps, and validation techniques are provided.

Keywords

Partial Wave Spectroscopic microscopy; chromatin structure; chromatin topology; nanoscale imaging; fractal dimension; cancer development; heterogeneity; live cell imaging; transcriptional

1. Introduction

1.1 Chromatin heterogeneity

Chromatin is a complex nuclear structure that comprises the genome including DNA, histones, RNA, and other nuclear proteins. Many of the most important cellular functions, such as DNA transcription, replication, and repair, occur within the chromatin structure. The organization of chromatin occurs across a range of length scales from a few nanometers up to the micron range. These changes are shown to have regulatory roles in these molecular functions. At the smallest length scales, the DNA molecule (1 nm) is wrapped around a core

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nucleosome particle (10 nm) whose regulatory role is mediated in part by post-translational modification of the histone proteins. Above this length scale, chromatin is further compacted into a complex organization that is poorly understood (supranucleosomal structure). Studies utilizing chromatin conformation capture techniques have shown that between 100-300 nm chromatin is organized into topologically associated domains, where genes within the same domain interact more frequently compared to adjacent regions [1]. At the largest length scales (>300nm), chromatin is organized into chromatin territories, which can be resolved by visible-light diffraction-limited microscopy methods including confocal microscopy and wide-field fluorescence microscopy. Despite extensive research in this area, current understanding of chromatin organization in a range between 10 and 200 nm is still limited. Label-free visible-light optical microscopy techniques are optimal tools for studying cellular structures because they can image live cells in a non-invasive and non-toxic manner, but they are unable to resolve structures smaller than the diffraction limit (~200nm). To probe these length-scales, most methods to date rely on indirect measurements using chromatin conformation capture (3C, 4C, HiC, etc.) or direct measurements using electron microscopy, but these techniques involve intensive sample preparation and fixation, preventing temporal analysis of a cell in response to perturbation and potentially introducing artifacts. Therefore, non-invasive microscopic methods that are sensitive to structural alterations at these intermediate length scales are still lacking.

Notably, the organization of chromatin at these length scales (higher-order chromatin topology) defines the physical context (nanoenvironment) in which biological processes occur. In general, research into the aforementioned processes, such as DNA transcription or replication, has taken place in *ex vivo* dilute conditions, a context vastly different from what is found within cells. When the effects of the nanoenvironment are measured and taken into consideration, they demonstrate a critical functional role in the regulation of chemical reactions. For instance, multi-scale systems modeling using molecular dynamics simulations has shown that the physical environment, through macromolecular crowding, non-linearly alters gene transcription by orders of magnitude[2]. Additionally, *in vivo* studies have shown that local nuclear density will slow the diffusion of macromolecules[3]. Finally, macromolecular crowding has been shown to affect protein binding stability and enzyme structure [4]. Thus, the capacity to measure and analyze the physical nanoenvironment modulated by higher-order chromatin folding can increase our understanding of disease processes and molecular behaviors.

To study this physical nanoenvironment, we have developed a novel imaging technique, Partial Wave Spectroscopic (PWS) microscopy, which allows real-time measurement of chromatin structure (chromatin folding) below the diffraction limit without introducing labels in live cells[5]. PWS microscopy allows one to measure variations in the interference spectrum of the backscattered light and to quantify heterogeneity in the structural organization within cells at the nanoscale level [6]. This capability derives from the interaction between light and intracellular mass. In particular, the wavelength-dependent variations in scattered light are determined by the density and distribution of molecules with sensitivity to macromolecular assemblies below the diffraction limit – i.e. higher order chromatin in the nucleus. Analogously, while our eyes cannot resolve the micron-scale particles that compose clouds, we can obtain information on the size and distribution of

particles when comparing the color of white clouds to the blue sky. When using PWS microscopy the variations in mass density within a sample are quantified through Σ (Fig 1AB)[5], which is a measure of intracellular nanoscale heterogeneity[6]. Chromatin heterogeneity refers to variations in structural density within the higher order chromatin organization. Specifically, increases in heterogeneity result in an organization that is globally accessible paired with highly dense, local clumps of poorly accessible chromatin. As a visual example of what PWS microscopy measures, chromatin was simulated as a 10nm “beads on a string” polymer with equivalent mass density, but different nanoscale organizations: differentially compacted (Fig. 2A)[5] and homogeneously compacted (Fig. 2B) [5] chromatin fibers. The resulting diffraction limited transmission microscopy images (Fig. 2CD)[5], and Σ images (Fig. 2EF)[5] were calculated directly from the mass distribution of the simulations. The differences between these two configurations are nanoscale, so they cannot be resolved or sensed with the transmission microscope, but as a physical measure of heterogeneity at these length scales, PWS microscopy quantifies these differences with the heterogeneously organized chromatin producing a high- Σ image compared to the homogeneously organized chromatin.

1.2 Fractal Dimension

Intracellular nanoscale heterogeneity, measured using PWS, is directly correlated to the fractal dimension, D [6]. Fractal dimension defines the self-similarity of organization across a given range of length scales (the geometric relationship of a single nucleotide to a nucleosome to a fiber, and so on) and is related to the space-filling capacity of a particular geometry. The organization of chromatin has been suggested to be fractal with dimension D using multiple techniques[7], such as small angle neutron scattering[8], fluorescence correlation spectroscopy[3], chromatin conformation capture[9], transmission electron microscopy[10,11], and photon localization microscopy[12,13]. It should be noted that the organization of chromatin as a fractal media is not synonymous with the fractal globule model. The fractal globule model is a specific case of a fractal structure with dimension $D=3$ [14], however, the power-law (fractal) scaling can be achieved by a random-loop polymer or a ‘fractal globule’. Indeed, while the underlying scaling of chromatin organization can be explained by any of these models, all models converge on a self-similar geometry. Within chromatin, D is a measure of the structural heterogeneity. Increases in D will lead to an increase in chromatin surface area and variations in local density [15]. By increasing the chromatin surface area, DNA become more accessible to transcription factors. Conversely, increasing the heterogeneity of local density produces clumps of dense, inaccessible DNA. As these divergent responses are linked, it was theorized that a larger D leads to a divergence (or heterogeneity) in gene expression, where active genes become overexpressed, while repressed genes become further underexpressed [15]. We have demonstrated using PWS microscopy and microarray analysis that changes in Σ result in global alterations in the pattern of gene expression, influencing critical cellular functions such as metabolism, proliferation, transcriptional regulation, signaling cascades, and cellular development [15]. As expected Σ was correlated with the heterogeneity of gene expression, showing an overexpression of initially active genes, and an underexpression of initially repressed genes as Σ increases [15]. Additionally, while the current consensus indicates that the spatial correlation function of chromatin density distribution follows power-law scaling, these

results and our interpretations of PWS measurements are still valid under conditions where chromatin organization is non-fractal. Regardless of the nature of chromatin folding, the organization of chromatin can be described by a spatial correlation function of its density in the form of the Whittle-Matern family of functions [6]. In this approach, the scaling relation D remains proportional to the molecular correlation distance even for non-fractal media and quantifies the self-similarity of chromatin organization. As a result of the correlation between Σ and D , PWS can be used to explore the relationship between higher-order chromatin structure and global changes in gene expression.

1.3 The Role of Chromatin Heterogeneity in Cancer

This divergence in gene expression in response to altered chromatin topology may play a role in the development of cancer. This would not be a surprise as the dysregulation of gene networks, in particular, the inhibition of tumor suppressors and the activation of pro-growth oncogenic pathways, are a hallmark of tumorigenesis. Histological (microscale) alterations in the nuclear structure, such as size, shape, chromatin texture, nuclear matrix, etc. have been a gold standard of tumor identification across multiple types of cancer [16,17]. In addition to the typical histological markers, an increased microscale fractal dimension of chromatin has been detected across different cancer types and shows potential prognostic value [18–21]. Further, transmission electron microscopy (TEM) has identified increases in nanoscale chromatin fractal dimension at an early stage prior to any histologically detectable changes in colorectal cancer (CRC) animal models and human samples from patients with colorectal adenomas [11]. Fixed cell PWS microscopy has detected these same structural changes, which extend to a variety of cancers: colorectal, lung, pancreas, ovarian, esophagus, and prostate [22–27]. Furthermore, these alterations detected using TEM and PWS microscopy are observed throughout the entire organ, not just at the location of the lesion. This is due to field carcinogenesis which is the concept that diffuse molecular and structural alterations exist in healthy tissue prior to the development of a localized tumor. These results suggest that measuring chromatin topology in patient samples using PWS microscopy has promising potential as a tool for cancer screening.

These findings indicate that increased chromatin heterogeneity is an early and near-universal step in the development of cancer regardless of the underlying tissue or molecular pathway. Mechanistically, the role of a more heterogeneous chromatin topology in early carcinogenesis could be related to increasing the heterogeneity of gene expression, thereby enabling greater sampling of the genome - an advantageous survival response to repeated environmental stress conditions [28,15]. Over time there will be an evolutionary selection for a population of cells with the greatest capacity to explore their genome, a higher tolerance to environmental stress, a broad distribution of initial cells states, and ability to delay commitment to apoptosis; these are features which may facilitate tumor formation [28].

1.4 PWS Microscopy Theory

The ability of PWS microscopy to probe intracellular nanoscale organization derives from the relationship between the distribution of mass density, refractive index, the scattering of light, and the interference of light. Mass density and refractive index are related by the Gladstone-Dale relationship $n = n_w + \alpha \rho$, where n_w is the refractive index of water, α is the

specific refractive index increment (0.18 ml/g), and ρ is the cell density. Light incident upon cells will be weakly scattered due to variations in refractive index arising from the distribution of mass/macromolecular structures. Within our system, light interference is utilized to enhance the signal from this weakly scattered light.

Specifically, cells are illuminated from below and the light reflects from two main sources: 1) the strong reflection at the glass-cell interface due to the large refractive index (RI) difference (referred to as the reference reflection), and 2) the weakly scattering light from within the cell due to RI variations (referred to as internal scattering) (Fig. 3). These two signals interfere, amplifying the scattering signal and resulting in spectral intensity variations, deriving from the distribution of RI with a sensitivity to length-scales between 20 and 200nm.

In more detail, if we assume an inhomogeneous sample with RI distribution $n_1[1+n(r)]$ as a function of location r (Fig. 3) is placed into the focal plane of our microscope, then for the spectral variance of the image intensity, $\Sigma^2(x,y)$, within the measured wavenumber range, k , will be related to the organization of refractive index within the sample as

$$\frac{E[\Sigma^2]}{k_c L} = \frac{R k_c}{\Delta k} \int_{T_{3D}} \Phi_{n_\Delta}(k) d^3 k \propto \sigma_{n_\Delta}^2 l_c$$

, where $E[\Sigma^2]$ is the expected value of Σ^2 , $\Phi_{n_\Delta} = |\mathcal{F}\{n_\Delta(r)/V\}|^2$ is the power spectral density of the sample RI variations n normalized by sample volume V , k_c is the central wavenumber of the illumination bandwidth evaluated inside the sample, L is the sample thickness, $R=R_{01}T_{01}T_{10}$ is an intensity normalization based on the Fresnel transmission (T_{01} , T_{10}) and reflection (R_{01}) intensities at the sample interfaces, T_{3D} includes spatial frequencies k with longitudinal coordinates k_z between $2k_1$ and $2k_2$ (k_1 and k_2 are the lower and upper wavenumbers of the instrument bandwidth) evaluated within the sample, σ_n is the standard deviation of RI fluctuations, and l_c is the RI correlation length [6,29].

In short, Σ is a measure of the distribution of mass density within the sample. It is proportional to the variance of molecular density and the correlation length of the molecular density arrangement within the sample. The correlation length, l_c , describes the distribution of different length scales present in the sample. Furthermore, σ_n and l_c are both proportional to the heterogeneity of the RI distribution.

1.5 PWS Instrument

A diagram of the live cell PWS instrument can be seen in Figure 4[5]. A broadband non-coherent white light (from X-Cite 120LED) is passed in through the episcopic port of a commercial microscope base (Leica DMIRB) with a low illumination NA. Then the light enters the filter cube, where a UV blocking filter is used to remove harmful wavelengths for live cell imaging, or alternative filters can be used for fluorescence colocalization. A high magnification oil immersion objective (63× Leica HCX PL APO, NA 0.6-1.4) focuses the

light onto the sample. The backscattered light is collected and spectrally filtered (CRi VariSpec LCTF) across a range of wavelengths while imaging with a scientific camera (Hamamatsu Image-EM CCD) at each wavelength.

PWS instrumentation can be constructed in a variety of ways assuming the design criteria described above are met. Any non-spatially coherent broadband visible light source is viable if it has a relatively flat emission spectrum in the visible range and low temporal variations. Spectral filtration can be performed on either the illumination or collection side of the system, although in a live cell instrument filtration on the illumination-side is preferred to minimize power at the sample. Another potential benefit of illumination side spectral filtration is that it can eliminate the need for additional filters to remove potentially damaging wavelengths (UV/IR) from the source spectrum before the sample. In addition to the location of the spectral filter, multiple methods of filtration can be used depending on the experimental goals of the system. On the illumination side an acousto-optic tunable filter (AOTF) or tunable monochromator works well, and on the collection side, a liquid crystal tunable filter (LCTF) or spectrometer can be used. These different filter methodologies provide options depending on the desired system complexity, speed, spectral resolution, and ability to perform experiments with other optical imaging modalities on the same hardware. Finally, any low-noise, high-sensitivity scientific camera such as an EMCCD or sCMOS designed for fluorescence microscopy should be suitable for image collection. The choice of camera and objective magnification should ideally provide a resolution of approximately two pixels per diffraction limited spot for an accurate spatial sampling of the spectra.

1.6 Fluorescence Colocalization

As a multimodal system, the PWS microscope can also acquire fluorescence images for colocalization or other biological tests to combine molecular specificity with sensitivity to nanoscale structures. Not only does this enable easy segmentation of nuclear Σ , but allows for further segmentation to measure the macromolecular assemblies for a variety of cellular organelles and functional domains (Fig. 5)[5]. Additionally, colocalization with fluorescently labelled proteins (e.g. HER2: GFP) enhances the ability of PWS to explore the important relationship between cellular structure and molecular function in live cells. We have demonstrated the capability to integrate PWS microscopy with confocal[30] and wide-field fluorescence microscopy[5], and integration with super-resolution imaging methods such as PALM/STORM, SIM, or STED is achievable.

1.7 Experimentally Observed Chromatin Alterations

PWS microscopy is an ideal tool to study real-time alterations in chromatin structure because it (i) quantifies structural organization at length scales (20-200nm) difficult to image with alternative techniques (ii) using label-free contrast and low imaging power to preserve cellular integrity and function, with (iii) high-throughput, real-time imaging speed, and (iv) the ability to track individual cells over time. As an example of experimentally observed chromatin alterations, we have treated HeLa cells with 2 μ M of daunorubicin for 15 minutes (Fig. 6). Daunorubicin is a chemotherapy drug that influences chromatin topology through multiple mechanisms, such as intercalation and nucleosomal eviction[31]. Through PWS imaging, we have observed that treatment with daunorubicin induces homogenization of the

chromatin structure [$33\pm 9\%$ (mean $\pm$ SD) decrease in Σ , population measurements comparing treated with untreated, $n=18$ experiments with ~ 30 cells in each group]. In the daunorubicin experiments, we have shown differences in chromatin topology between two population groups (treated vs. untreated).

As an example of using PWS to track chromatin alterations in individual cells, we have treated a triple-negative, mutant *TP53* breast cancer cell line, MDA-MB-231, with $5\mu\text{M}$ of the zinc chelator TPEN (Fig. 7). This treatment is expected to sequester intracellular labile zinc thus inducing a zinc-deficient state. Because of zinc's ubiquitous roles, the downstream effects of this treatment are likely multifold including a change in the concentration of ions in the nucleus, alteration of metalloprotein structure and activity, and disruption of key metabolic pathways. Under this treatment, there is an observed increase in the heterogeneity of chromatin within thirty minutes of treatment [$8.2\pm 1.2\%$ (mean $\pm$ SEM) decrease in Σ , individual cells tracked over thirty minutes, $n=24$ cells]. Last, we show that PWS makes it possible to track individual cells over time by continuously imaging live MDA-MB-231 cells over the course of thirty minutes (Video 1).

2. Materials

2.1 Reagents

2.1.1—Immersion oil (Cargille Immersion Oil, Type 37 for imaging at 37°C or LDH at room temperature)

2.1.2—Size 0, 1, or 1.5 sterile glass bottom imaging dishes (can be purchased from MatTek, CellVis, or World Precision Instruments) (see Note 1)

2.1.3—Mixed balance (N_2 , O_2 , and CO_2) gas tanks to maintain atmospheric conditions and pH for continuous cell growth. Formulation of the mix depends on the cell line of interest.

2.1.4—Cell media is determined by the cell type of interest (RPMI 1640, McCoy's 5A, DMEM, and many others are compatible with system measurements). Changes in phenol red absorption in pH dependent experiments can alter the spectra.

2.2 Equipment

2.2.1—Commercial microscope base (preferably inverted) with an episcopic light path (e.g. Leica DMIRB)

2.2.2—Broadband non-spatially coherent white light source (X-Cite 120LED)

It is ideal to select a source with a relatively flat output spectrum in the visible range ($\sim 400\text{--}700\text{nm}$) without any sharp peaks. It is also recommended that the instrument utilize a light source with minimal temporal variations.

2.2.3—High magnification oil immersion objective ($63\times$ Leica HCX PL APO, NA 0.6-1.4)

Objective and camera selected for imaging should result in a pixel size below the diffraction limit. (<250nm), ideally yielding two pixels per diffraction limited spot.

2.2.4—Spectral filter (CRi VariSpec LCTF).

Many varieties are viable including: AOTF, LCTF, tunable monochromator, or spectrometer. Can be placed in illumination or collection path depending on system design goals and filter type chosen

2.2.5—Scientific camera such as an EMCCD or sCMOS (Hamamatsu Image-EM CCD)

sCMOS is recommended for higher speed imaging and a larger field of view unless other modalities being performed require the sensitivity of an EMCCD.

2.2.6—Automated stage.

Allows for automatic collection of multiple fields of view.

2.2.7—Autofocus system is recommended for long time series (>30minutes) experiments.

2.2.8—Automated shutter.

Can be used to automatically block light when not imaging to avoid damaging cells for multiple hour experiments (*See Method Cell Stability*)

2.2.9—Thin film phantoms (Filmetrics Inc.)

Can be used for validation experiments (*See Method System Validation: Phantoms*)

2.2.10—Fluorescence filters

2.2.11—UV filter. UV irradiation damages cells through multiple pathways, such as DNA damage[32], membrane permeabilization[33], and altered mitochondrial behavior[34]. It is advisable to block all UV light (100nm – 400nm) from illuminating the cells. If the tunable spectral filter is placed on the collection side of the instrument, then a UV filter must be added to the illumination side to prevent cell damage. If the tunable spectral filter is placed on the illumination side, then no extra filter is necessary, but you must avoid tuning the spectral filter to UV wavelengths during acquisition.

2.2.12—Live cell incubator.

2.2.13—Computer.

3. Methods

3.1 System Set Up

3.1.1—Turn on the digital camera source and allow the requisite time for heating up before proceeding. Systems that require cooling, should achieve a stable temperature before measurements begin. Then turn on light source and automated stage controller.

3.1.2—Set up the live cell incubated chamber if not already configured. Some cell lines require the use of a mixed balance 21% O₂, 5% CO₂, Balance N₂. Prior to imaging, ensure a proper flow to the cells to produce a stable environment in the incubated chamber. Check to make sure the internal temperature of the chamber is stabilized at 37°C unless the experiment requires an alternate temperature (*see Note 2*).

3.1.3—Set the microscope to episcopic/reflection mode.

3.1.4—Set the filter cube to UV blocking filter (if necessary) to protect the live cells from damage.

3.2 Preparing Samples

3.2.1—Using standard culture conditions and media requirements as applicable to the cell line of interest, grow cells on a glass-bottom petri dish until they are about 50 to 70 percent confluent. Cells can be observed using a separate transmission microscope to ensure proper density and morphology. Some cell lines require additional plating time to stabilize; in general we recommend at least 24 hours after splitting to allow cells to adhere and acclimate before experimentation. These experimental protocols are designed to be used with adherent cell lines. Caution should be used with non-adherent cell lines, as changes in sample geometry (Fig. 3), cell motion, etc. can interfere with the PWS signal.

3.2.2—Switch to the oil immersion objective of desired magnification. Place a drop of immersion oil on top of the objective, and then place the dish in the microscope incubation chamber. Course adjust the objective focus until oil contacts the glass bottom of the dish. Allow at least 15 minutes for the oil to reach thermal equilibrium in order to avoid focal drift during acquisition.

3.2.3—Tune the spectral filter to the central wavelength of the imaging range to focus on the cell imaging plane of interest (*see Note 3*).

3.3 Measuring Cellular Heterogeneity

3.3.1—Using either the microscope eyepieces or computer software with live feed camera display, fine adjust the objective focus until cells and/or substrate is in focus for a fixed wavelength. See Note 4 if a clear focused image cannot be obtained.

3.3.2—Locate a region in the dish without any cells or debris for a normalization measurement. Normalization accounts for changes in the lamp, camera, and cell media prior

to imaging. If a clear region cannot be located, a clean dish with identical cell media can be used for the normalization measurement.

3.3.3—Readjust focus as necessary.

3.3.4—Tune the spectral filter to scan the filtration wavelength through the chosen wavelength range, and sync the camera to collect an image at every wavelength. This configuration will allow for a collection of an interference spectra. See Note 5 for details on setting up imaging parameters.

3.3.5—This interference spectra data should be saved as a 3-D image cube of dimensions x-pixel-range by y-pixel-range by wavelength range (x,y, λ). It is helpful to automatically save additional imaging parameters such as date, time, exposure time, lamp power, wavelength range and step size, stage position, etc.

3.3.6—Locate a field of view with desired cells to image and readjust focus if necessary.

3.3.7—Once located and focused, acquire the cellular interference spectra (3-D image cube). Make sure to record imaging location if you plan to reimage the same cells after treatment or later in the experiment.

3.3.8—It is best practice to analyze the first image cube to check for any artifacts before proceeding with the experiment (*see* Note 6).

3.3.9—Repeat steps 6 and 7 as many times as needed to reach desired sample size.

3.4 Colocalization with Fluorescence (see Note 7)

3.4.1—Locate a field of view with desired cells to image and readjust focus if necessary.

3.4.2—Switch the filter cube to the appropriate filter for the fluorescence dye in use.

3.4.3—Tune the spectral filter to sweep the filtration wavelength based on the emission spectra of the fluorophore. Note, systems with multiple camera ports can utilize a second camera without a spectral filter for co-localization. Alignment between the two cameras can be made with a standard fiduciary marker.

3.4.4—Set the camera to capture an image at each wavelength, average those images together, and save the resulting fluorescence image.

3.5 Data Analysis

3.5.1—Subtract the dark counts of the digital camera from every point in the collected image cubes and normalization cube (*see* Note 8).

$$\text{image_cube}(x,y,\lambda) = \text{image_cube}(x,y,\lambda) - \text{dark counts}$$

3.5.2—If exposure times varied, divide every point in the collected image cubes and normalization cube by the exposure time.

$\text{image_cube}(x,y,\lambda) = \text{image_cube}(x,y,\lambda) / \text{exposure time}$

3.5.3—Divide the image cube of cell measurements by the normalization cube.

$\text{image_cube} = \text{image_cube} / \text{normalization_cube}$

3.5.4—Use a low-pass filter across the spectra at each x-y pixel in the normalized image cube to eliminate high-frequency noise.

- The cutoff frequency should be set to remove spectral frequencies that do not originate from the sample. First, there cannot be frequencies above the spectral resolution of the spectral filter being used. Also, the theoretical frequencies expected from the sample can be calculated from sample thickness and imaging parameters (*see Note 5*). Alternatively, this can be determined by checking the spectra (*see Method Examining Spectra*).

3.5.5—To calculate Σ at each x-y pixel, take the standard deviation of the filtered, normalized image cube across the wavelength range. This will produce the 2-D matrix, $\Sigma(x,y)$

3.5.6—Perform region-of-interest analysis for each Σ image to capture average Σ of whole cells or cellular compartments (e.g. nuclei) (*see Note 9*). All operations may be performed in MATLAB.

3.6 Cleaning Up System

3.6.1—Remove the dish from the incubated chamber and properly dispose of.

3.6.2—Turn off the mixed balance tank and ensure flow stabilizes at 0 CCM.

3.6.3—Clean oil off the objective using lens paper with a few drops of methanol. Be very gentle to avoid damaging the objective.

3.6.4—Turn off the light source, manual stage controller, and digital camera source immediately.

3.7 Examining Spectra

Checking both the raw and normalized spectra is a good way to diagnose issues with the system or individual data.

3.7.1—Normalized Spectra: To examine the normalized spectra, perform the analysis procedure through the filtration step (#4). Then, plot the intensity as a function of wavelength at individual pixels to view the interference spectra (Fig. 6AB).

Individual Pixel Spectra. Ideal spectra at individual pixels should vary from point to point and contain a variety of frequencies. If the spectra does not vary from point to point (in an inhomogeneous sample like a cell), then the instrument is not collecting the interference signal or it is being dominated by an undesired signal. The spectra should contain all frequencies in k-space up to $\frac{n_1 * L}{\pi}$, where n_1 is RI of the sample, and L is the sample thickness. Spectra from within the cell can be compared to background spectra to determine the frequencies that come from the sample versus noise. Noise should be filtered out in post processing.

Averaging Spectra. Multiple individual spectra can be averaged over an area large enough to contain multiple diffraction-limited spots to observe the mean spectra. This averaging should remove the interference information. An ideal mean spectra should be relatively flat compared to the individual spectra. In regions without cells, the normalized intensity should be equal to one (image_cube intensity equal to normalization_cube intensity). The standard deviation of the mean spectra can be compared to individual interference spectra to check how much of the signal is coming from interference versus other unwanted sources. Any spikes or consistent features of the mean spectra may be due to issues such as switching artifacts from the spectral filter, improper timing/delays of the spectral filter, and absorption or fluorescence from dyes.

Checking the spectra from phantoms (*see Method System Validation: Phantoms*) is a useful technique for validating your instrument. It should be noted that these phantoms are likely to be homogenous, so it is normal if the individual pixel and averaged spectra are identical.

3.7.2—Raw Spectra: No analysis is required to view the raw spectra. Just plot the intensity from your image_cube or normalization_cube as a function of wavelength at individual pixels (Fig. 6AC). Raw spectra mostly provide information on the lamp profile, which is useful for determining the wavenumber range used for imaging. Avoid using regions in the lamp spectra with low signal. Raw spectra are also useful for identifying saturation regions of your spectra. These regions can be identified by looking for totally flat and maximized regions of the spectra. If the camera is saturating, the exposure time or lamp power must be reduced. Finally, raw spectra can be used to determine whether artifacts found in the normalized spectra are due to the image_cube or the normalization_cube or both as the spectra can be viewed individually.

3.8 System Validation: Phantoms

Phantoms can be used to provide a standard for system validation. The simplest phantoms are measurements taken from surface reflections with different theoretical reflection intensities, such as glass-air, glass-water, glass-glycerol, sapphire-air, silicon-air, etc. RI matching liquids (Cargille Laboratories) can be used to create a large variety of interfaces. The expected reflectances can be calculated with Fresnel equations. These tests can show extra reflections in the system, dirty lens/objectives, improper analysis normalization, and other issues. Care should be taken to match experimental and theoretical reflectance values

in a wavelength-dependent manner since the wavelength-dependent precision of the instrument is crucial for PWS measurements.

Filmetrics, Inc. provides a variety of thin film thickness standards that produce a stable and well characterized uniform interference signal. The measured spectral interference frequencies can be compared to theoretically calculated frequencies to validate your system. In addition to validation, these phantoms also provide a good standard to compare between different systems, and to check your system's performance stability over time or after instrumentation changes.

3.9 System Validation: Live Cell Delta

One useful practice to ensure stability in the system and cell lines is to monitor a consistent structural alteration. The daunorubicin treatment (HeLa cells with 2 μ M of daunorubicin for 15 minutes) previously discussed is a good experiment to use for this validation. While further work is needed to unravel the molecular basis of this alteration, it is stable and can be used to validate/monitor system sensitivity to chromatin structural alterations in many contexts: over time, after system changes, through cell line passages, after different cell preparation protocols, etc.

3.10 Cell Stability

Cell stability should be tested to ensure that the system, imaging parameters, and experimental design are not affecting your data. Acquire PWS images of the same cells without treatment over the duration of a typical experiment to check that Σ is stable over time. It is normal for Σ to fluctuate in time as chromatin structure is dynamic, but there should not be a directional change. If Σ is not stable, it may be due to too bright illumination of the specimen or improper cell incubation/handling. There are a few solutions if lamp intensity is affecting the cells:

- Minimization of the light on cells can be achieved by employing spectral filtering at the illumination source instead of filtering at the collection side of the instrument.
- Reducing the duration of the experiment can help.
- Make sure proper UV filtering is being implemented.
- An automatic shutter can be used to block the light whenever measurements are not performed.

3.11 Using ImageJ to Create Movies

1. Acquire PWS data of the same field of view over desired period of time.
2. Analyze and output Σ images into a folder with sequentially numbered file names.
3. Open ImageJ and select File-Import-Image Sequence.
4. Choose appropriate folder and change Sequence Option if desired.

5. Select File-Save As-AVI.
6. Set a number of frames per second and compression (if desired).
7. Save movie file.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

4. Notes

1. Sample Geometry and Substrates

To achieve the proper PWS interference signal, the sample geometry must contain one and only one strong reference reflection. For live cell PWS, this is produced by the cell-glass interface (RI mismatch: 1.52–1.38), while the cell-media interface (RI mismatch: 1.38–1.33) results in a weak reflection (Fig. 3). For fixed cell PWS, the reference reflection is produced by the cell-air interface (RI mismatch: 1.0–1.53), while the cell-glass interface (RI mismatch: 1.53–1.53) produces almost no reflection. If the sample or substrates are changed, be sure this requirement is satisfied.

In general, glass-bottom imaging dishes are used because they are affordable and compatible with our imaging parameters, but alternative substrates such as sapphire can be implemented to increase the strength of the reference, resulting in an increase in SNR. On the other hand, a weaker reference reflection will reduce the SNR of your system.

2. Temperature Stabilization

It's preferable to have the temperature stabilize around 36 to 37°C. If the temperature has stabilized below this range, then check that the set point temperature is properly set to 37°C, ensure the incubator window is closed, and wait a few minutes. If the temperature has stabilized above this range, then check the configurations of the system. For experiments testing the influence of thermal stress (heat shock conditions), allow sufficient time for thermal expansion and stabilization of imaging oil.

3. Scanning Wavelength

Searching for cells and adjusting focus with the spectral filter tuned to the central wavelength is ideal because it minimizes focal drift due to chromatic aberrations. If the maximum lamp intensity occurs at a different wavelength, it is a good idea to check this wavelength to ensure the camera is not saturating with the current exposure time. Saturation of signal at any wavelength will result in improper measurements of the interference signal.

4. Blurry Image, Focus Issues, etc

If you are having trouble focusing/getting a clear image, there are a few easy-to-solve culprits:

- a. Water or condensation on the bottom of the petri dish can cause artifacts like blurry images. Clean off the bottom of the imaging dish and objective with methanol before applying new oil to the objective and starting again.
- b. Air bubbles in the immersion oil will cause large dark shapes in the field of view. These can be avoided simply by moving to a new area. If that doesn't solve the problem, clean off the bottom of the dish, clean the objective, add new oil to the objective, and start again.
- c. If the glass on the bottom of your petri dish is thicker than the working distance of the objective, then you will need different dishes. Typically, size 0, 1, or 1.5 glass will work with most objectives. The working distance should be a specification provided when purchasing your objective.

5. Imaging Parameters

Before taking measurements, imaging parameters must be set, which will vary based on your sample, system, and experimental design. There are four goals to consider when determining imaging parameters. First, you should capture all the spectral frequencies produced by your sample. The signal should contain all frequencies in k-space up to $\frac{n_1 * L}{\pi}$, where n_1 is the RI of the sample, and L is the sample thickness. Second, the SNR of your signal should be maximized. Third, you want to avoid introducing artifacts into the measurement. Finally, the measurement time should be minimized to increase efficiency, and reduce light exposure on the cells.

Step Size: The chosen step size must be small enough to resolve the highest spectral frequency produced by your sample. The minimum requirement, based on the Nyquist criterion, says that the sampling rate must be greater than twice this frequency; therefore the step size in k-space should be smaller than $\frac{\pi}{2 * n_1 * L}$. Imaging close to this minimum rate will reduce the measurement SNR. We recommend a step size $\leq \frac{\pi}{4 * n_1 * L}$.

Filtration Bandwidth: The bandwidth of the spectral filter will act as a low pass filter. In order to avoid filtering out sample frequencies, the bandwidth should be narrower than $0.5 * \text{step size}$. The bandwidth will also affect the SNR. While bandwidths larger than this limit will increase light intensity, it will actually reduce SNR by filtering out sample frequencies. Bandwidth smaller than this limit will reduce SNR by decreasing the collected intensity.

Wavenumber Range: In order to capture at least one full oscillation from our highest frequency, the wavenumber range, k , must be greater than $\frac{\pi}{n_1 * L}$. This is a very minimal requirement. If the range is set to this exact limit, none of the lower frequency information will be captured, and SNR will be significantly reduced. Ideally, the largest range possible will produce the best SNR, but it will be at the expense of imaging time. Additionally, there will be limitations based on the intensity profile of your lamp and the efficiency profile of

your spectral filter. You should avoid spectral regions with low output based on these equipment specifications. Finally, you will want to avoid irradiating your cells with spectra known to cause cell damage, in particular, UV light.

Exposure Time: An ideal exposure time is a tradeoff between SNR and measurement time. The most important requirement for exposure time is to avoid saturating the camera at any wavenumbers (see **Method Examining Spectra**).

Lamp Power: Lamp power can also be increased to improve SNR, but should not be so strong as to damage the cells or saturate the camera (see **Methods Examining Spectra** and **Cell Stability**).

Spectral Filter Delays: Proper delays must be set between each image when tuning the spectral filter to ensure the filter is stabilized at each wavelength. Improper delays will introduce spectral artifacts.

6. First Image Cube Check

It is best practice to analyze the first image cube to check for any artifacts before proceeding with the experiment. Unexpected lamp fluctuations, system vibrations, floating debris, and other various issues can interfere with proper measurements and experimental findings. If there is an issue with the normalization measurement, the artifact will propagate through all your data. It is best to catch these errors early so that the measurements can be retaken.

7. Colocalization with Fluorescence

For optimal colocalization, each fluorescent image should be acquired directly after each PWS measurement before moving the stage. This may not always be possible depending on your staining protocol (e.g. immunofluorescence). A 2nd camera can be used, or the spectral filter can be removed to achieve higher quality fluorescence images. If fluorescence images cannot be taken directly after PWS measurements or a secondary camera is used, image alignment will need to be performed using a fiduciary marker if accurate colocalization is required.

Caution should be used when designing experiments and choosing fluorophores. In general, most fluorophore absorption/emission is negligible relative to the backscattered intensity that it will not interfere with PWS signal, but this should be confirmed for each experimental design. Another concern is structural and functional alteration due to staining prior to the acquisition of PWS data. We have shown that Hoechst 33342, a commonly used live cell DNA binding dye, will cause DNA fragmentation within seconds of excitation[5]. Acquiring PWS data prior to cell staining will preserve the integrity of the PWS data, but structural and functional alteration due to preparation procedures (e.g. cell fixation) can still interfere with the interpretation of colocalization data.

8. Dark Counts

Camera dark count should be reported in the camera literature. Additionally, this can be measured by recording counts with the camera turned on and all light sources turned off.

9. Regions-of-interest Analysis

Region-of-interest (ROI) analysis can be performed on a single wavelength image from each cube. If nuclei are not clearly visible, it can be helpful to acquire colocalized phase, transmission, or fluorescence images to create ROIs. Fluorescence colocalization is particularly useful as heterogeneity in structures other than the nucleus can be obtained. While performing region-of-interest analysis, users may find it helpful to refer to the corresponding bright field image to more accurately locate the boundaries of cell membranes and cell nuclei.

References

1. Sexton T, Yaffe E, Kenigsberg E, Bantignies F, Leblanc B, Hoichman M, Parrinello H, Tanay A, Cavalli G. Three-dimensional folding and functional organization principles of the *Drosophila* genome. *Cell*. 2012; 148(3):458–472. DOI: 10.1016/j.cell.2012.01.010 [PubMed: 22265598]
2. Matsuda H, Putzel GG, Backman V, Szleifer I. Macromolecular crowding as a regulator of gene transcription. *Biophys J*. 2014; 106(8):1801–1810. DOI: 10.1016/j.bpj.2014.02.019 [PubMed: 24739179]
3. Bancaud A, Huet S, Daigle N, Mozziconacci J, Beaudouin J, Ellenberg J. Molecular crowding affects diffusion and binding of nuclear proteins in heterochromatin and reveals the fractal organization of chromatin. *The EMBO Journal*. 2009; 28(24):3785–3798. DOI: 10.1038/emboj.2009.340 [PubMed: 19927119]
4. Batra J, Xu K, Qin S, Zhou HX. Effect of macromolecular crowding on protein binding stability: modest stabilization and significant biological consequences. *Biophys J*. 2009; 97(3):906–911. DOI: 10.1016/j.bpj.2009.05.032 [PubMed: 19651049]
5. Almossalha LM, Bauer GM, Chandler JE, Gladstein S, Cherkezyan L, Stypula-Cyrus Y, Weinberg S, Zhang D, Thusgaard Ruhoff P, Roy HK, Subramanian H, Chandel NS, Szleifer I, Backman V. Label-free imaging of the native, living cellular nanoarchitecture using partial-wave spectroscopic microscopy. *Proceed Nat Acad Sci USA*. 2016; 113(42):E6372–E6381. DOI: 10.1073/pnas.1608198113
6. Cherkezyan L, Zhang D, Subramanian H, Capoglu I, Taflove A, Backman V. Review of interferometric spectroscopy of scattered light for the quantification of subdiffractional structure of biomaterials. *BIOMEDO*. 2017; 22(3):030901–030901. DOI: 10.1117/1.JBO.22.3.030901
7. Bancaud A, Lavelle C, Huet S, Ellenberg J. A fractal model for nuclear organization: current evidence and biological implications. *Nucleic Acids Res*. 2012; 40(18):8783–8792. DOI: 10.1093/nar/gks586 [PubMed: 22790985]
8. Lebedev DV, Filatov MV, Kuklin AI, Islamov AK, Kentzinger E, Pantina R, Toperverg BP, Isaev-Ivanov VV. Fractal nature of chromatin organization in interphase chicken erythrocyte nuclei: DNA structure exhibits biphasic fractal properties. *FEBS Lett*. 2005; 579(6):1465–1468. <http://dx.doi.org/10.1016/j.febslet.2005.01.052>. [PubMed: 15733858]
9. Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science*. 2009; 326(5950):289–293. DOI: 10.1126/science.1181369 [PubMed: 19815776]

10. Wu W, Radosevich AJ, Eshein A, Nguyen T-Q, Yi J, Cherkezyan L, Roy HK, Szleifer I, Backman V. Using electron microscopy to calculate optical properties of biological samples. *Biomed Optics Express*. 2016; 7(11):4749–4762. DOI: 10.1364/BOE.7.004749
11. Cherkezyan L, Stypula-Cyrus Y, Subramanian H, White C, Dela Cruz M, Wali R, Goldberg M, Bianchi L, Roy H, Backman V. Nanoscale changes in chromatin organization represent the initial steps of tumorigenesis: a transmission electron microscopy study. *BMC Cancer*. 2014; 14(1):189. [PubMed: 24629088]
12. Dong B, Almassalha LM, Stypula-Cyrus Y, Urban BE, Chandler JE, Nguyen T-Q, Sun C, Zhang HF, Backman V. Superresolution intrinsic fluorescence imaging of chromatin utilizing native, unmodified nucleic acids for contrast. *Proceed Nat Acad Sci USA*. 2016; 113(35):9716–9721. DOI: 10.1073/pnas.1602202113
13. Boettiger AN, Bintu B, Moffitt JR, Wang S, Beliveau BJ, Fudenberg G, Imakaev M, Mirny LA, Wu C-t, Zhuang X. Super-resolution imaging reveals distinct chromatin folding for different epigenetic states. *Nature*. 2016; 529(7586):418–422. <http://www.nature.com/nature/journal/v529/n7586/abs/nature16496.html#supplementary-information>. DOI: 10.1038/nature16496 [PubMed: 26760202]
14. Mirny LA. The fractal globule as a model of chromatin architecture in the cell. *Chromosome Research*. 2011; 19(1):37–51. DOI: 10.1007/s10577-010-9177-0 [PubMed: 21274616]
15. Almassalha LM, Tiwari A, Ruhoff PT, Stypula-Cyrus Y, Cherkezyan L, Matsuda H, Dela Cruz MA, Chandler JE, White C, Maneval C, Subramanian H, Szleifer I, Roy HK, Backman V. The global relationship between chromatin physical topology, fractal structure, and gene expression. *Sci Rep*. 2017; 7:41061.doi: 10.1038/srep41061 [PubMed: 28117353]
16. Zink D, Fischer AH, Nickerson JA. Nuclear structure in cancer cells. *Nat Rev Cancer*. 2004; 4(9):677–687. [PubMed: 15343274]
17. Robbins, SL., Kumar, V., Cotran, RS. Robbins and Cotran pathologic basis of disease Saunders/. Elsevier; Philadelphia, PA: 2010.
18. Bedin V, Adam RL, de Sa BC, Landman G, Metze K. Fractal dimension of chromatin is an independent prognostic factor for survival in melanoma. *BMC Cancer*. 2010; 10:260.doi: 10.1186/1471-2407-10-260 [PubMed: 20525386]
19. Tambasco M, Costello BM, Kouznetsov A, Yau A, Magliocco AM. Quantifying the architectural complexity of microscopic images of histology specimens. *Micron (Oxford, England : 1993)*. 2009; 40(4):486–494. DOI: 10.1016/j.micron.2008.12.004
20. Tambasco M, Magliocco AM. Relationship between tumor grade and computed architectural complexity in breast cancer specimens. *Human Pathol*. 2008; 39(5):740–746. DOI: 10.1016/j.humpath.2007.10.001 [PubMed: 18439940]
21. Metze K. Fractal dimension of chromatin: potential molecular diagnostic applications for cancer prognosis. *Expert Rev Mol Diagn*. 2013; 13(7):719–735. DOI: 10.1586/14737159.2013.828889 [PubMed: 24063399]
22. Subramanian H, Roy HK, Pradhan P, Goldberg MJ, Muldoon J, Brand RE, Sturgis C, Hensing T, Ray D, Bogojevic A, Mohammed J, Chang JS, Backman V. Nanoscale cellular changes in field carcinogenesis detected by partial wave spectroscopy. *Cancer Res*. 2009; 69(13):5357–5363. DOI: 10.1158/0008-5472.can-08-3895 [PubMed: 19549915]
23. Damania D, Roy HK, Subramanian H, Weinberg DS, Rex DK, Goldberg MJ, Muldoon J, Cherkezyan L, Zhu Y, Bianchi LK, Shah D, Pradhan P, Borkar M, Lynch H, Backman V. Nanocytology of rectal colonocytes to assess risk of colon cancer based on field cancerization. *Cancer Res*. 2012; 72:2720–2727. DOI: 10.1158/0008-5472.CAN-11-3807 [PubMed: 22491589]
24. Konda VJ, Cherkezyan L, Subramanian L, Becker V, Goldberg MJ, Chennat JS, Karl LR, Waxman I, Roy LK, Backman V. Nanoscale differences assessed by partial wave spectroscopy in the field of esophageal cancer and Barrett's esophagus. *Gastroenterology*. 2011; 140(5):S752–S752.
25. Roy HK, Brendler CB, Subramanian H, Zhang D, Maneval C, Chandler J, Bowen L, Kaul KL, Helfand BT, Wang CH, Quinn M, Petkewicz J, Paterakos M, Backman V. Nanocytological field carcinogenesis detection to mitigate overdiagnosis of prostate cancer: a proof of concept study. *PLoS One*. 2015; 10(2):e0115999.doi: 10.1371/journal.pone.0115999 [PubMed: 25706755]

26. Damania D, Roy HK, Kunte D, Hurteau JA, Subramanian H, Cherkezyan L, Krosnjar N, Shah M, Backman V. Insights into the field carcinogenesis of ovarian cancer based on the nanocytology of endocervical and endometrial epithelial cells. *International Journal of Cancer*. 2013; 133(5):1143–1152. DOI: 10.1002/ijc.28122 [PubMed: 23436651]
27. Subramanian H, Pradhan P, Liu Y, Capoglu IR, Rogers JD, Roy HK, Brand RE, Backman V. Partial-wave microscopic spectroscopy detects subwavelength refractive index fluctuations: an application to cancer diagnosis. *Opt Lett*. 2009; 34(4):518–520. DOI: 10.1364/OL.34.000518 [PubMed: 19373360]
28. Almassalha LM, Bauer GM, Chandler JE, Gladstein S, Szleifer I, Roy HK, Backman V. The greater genomic landscape: the heterogeneous evolution of cancer. *Cancer Res*. 2016; 76:5605–5609. DOI: 10.1158/0008-5472.can-16-0585 [PubMed: 27550448]
29. Cherkezyan L, Capoglu I, Subramanian H, Rogers JD, Damania D, Taflove A, Backman V. Interferometric spectroscopy of scattered light can quantify the statistics of subdiffractive refractive-index fluctuations. *Physical Rev Lett*. 2013; 111(3):033903.
30. Chandler JE, Stypula-Cyrus Y, Almassalha L, Bauer G, Bowen L, Subramanian H, Szleifer I, Backman V. Colocalization of cellular nanostructure using confocal fluorescence and partial wave spectroscopy. *Journal Biophotonics*. 2016; 10:377–384. DOI: 10.1002/jbio.201500298 [PubMed: 27111884]
31. Yang F, Teves SS, Kemp CJ, Henikoff S. Doxorubicin, DNA torsion, and chromatin dynamics. *Biochim Biophys Acta*. 2014; 1845(1):84–89. DOI: 10.1016/j.bbcan.2013.12.002 [PubMed: 24361676]
32. Roos WP, Thomas AD, Kaina B. DNA damage and the balance between survival and death in cancer biology. *Nat Rev Cancer*. 2016; 16(1):20–33. DOI: 10.1038/nrc.2015.2 [PubMed: 26678314]
33. Schwarz T. UV light affects cell membrane and cytoplasmic targets. *J Photochem Photobiol B*. 1998; 44(2):91–96. DOI: 10.1016/S1011-1344(98)00126-2 [PubMed: 9757589]
34. Gniadecki R, Thorn T, Vicanova J, Petersen A, Wulf HC. Role of mitochondria in ultraviolet-induced oxidative stress. *J Cell Biochem*. 2000; 80(2):216–222. [PubMed: 11074592]

Figure 1. Representative Σ images of A) HeLa cells and B) Mes-SA cells quantify the heterogeneity of higher-order chromatin structure within label-free live cells. Σ scaled to range between 0.01 and 0.05 in A and 0.01 and 0.065 in B. Scale bar: 20 μm . Figure originally published in [5].

Figure 2.

Orthographic z-axis projection of molecular dynamics simulations of chromatin as a 10-nm “beads on a string” polymer with A) differentially compacted ($l_c = 70$ nm) and B) homogeneously compacted ($l_c = 20$ nm) chromatin fibers. Diffraction limited widefield microscopy representation of the simulated C) differentially compacted and D) homogeneously compacted chromatin fibers. These images were produced by calculating the average mass density at each pixel, and a Gaussian PSF of 250 nm was applied to simulate a conventional microscope. The grid size of the simulations was 10 nm. Σ images calculated

from the simulations of E) differentially compacted and F) homogenously compacted chromatin fibers. Σ was calculated directly from the distribution of mass within configurations shown in *A* and *B*. Σ scaled to range between 0.01 and 0.065. (Scale bar: 100 nm.) Figure originally published in [5].

Figure 3.

Diagram of the live cell PWS sample geometry. PWS microscopy requires one strong reflection, referred to as the reference. In our live cell PWS set up, the reference originates from the refractive index difference between the glass and cell. The internal scattering signal comes from the refractive index variations within the cell due to the organization of mass. PWS quantifies the higher-order organization of chromatin by measuring the spectral variations due to the interference between the reference and the internal scattering.

Figure 4.

Diagram of the PWS microscope. The system is built into an inverted commercial microscope base. The illumination is provided by a broadband white light source. The light scattered from within the cell is collected with an oil immersion objective, spectrally filtered with a liquid crystal tunable filter and imaged on a CCD camera. Figure originally published in [5].

Figure 5.

The live cell PWS microscope is a multimodal instrument capable of colocalizing A) PWS Σ images with B) widefield fluorescence microscopy showing mitochondria (green), nuclei (dark blue), and mitochondria-nucleus overlap (light blue). Σ scaled to range between 0.0125 and 0.065. Scale bar: 20 μm . Figure originally published in [5].

Figure 6.

Effects of daunorubicin on chromatin structure. A) Σ image of untreated HeLa cells. B) Σ image of HeLa cells treated with 2 μ M of daunorubicin for 15 minutes. Daunorubicin treatment induces homogenization of chromatin organization. C) Zoomed in Σ image of untreated nuclei. D) Zoomed in Σ image of daunorubicin treated nuclei. Scale bar: 5 μ m.

Figure 7. Effects of the zinc chelator TPEN on chromatin structure. A) Σ image of the untreated MDA-MB-231 cell. B) Quantification of average nuclear Σ normalized to the initial state from the same cells before treatment and after 30 minutes of treatment with 5 μ M TPEN. This TPEN treatment induces an increase in the heterogeneity of chromatin organization. C) Zoomed in Σ image of nuclei before treatment. D) Zoomed in Σ image of the same nuclei after 30 minutes of treatment. Scale bar: 5 μ m. **** p < 0.0001 using paired student's t-test.

Figure 8. Example spectra. A) Reflection image of cells showing the location of the graphed spectra B) These normalized individual spectra from the nuclei (purple and turquoise) show a variety of frequencies and vary between locations. The spectrum from the background (yellow) is flat across the spectral range. C) The raw spectra from the same locations is largely dominated by the lamp spectra.