What structural length scales can be detected by the spectral variance of a microscope image?

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A spectroscopic microscope, configured to detect interference spectra of backscattered light in the far zone, quantifies the statistics of refractive-index (RI) distribution via the spectral variance ($\tilde{\Sigma}^2$) of the acquired bright-field image. Its sensitivity to subtle structural changes within weakly scattering, label-free media at subdiffraction scales shows great promise in fields from material science to medical diagnostics. We further investigate the length-scale sensitivity of $\tilde{\Sigma}$ and reveal that, in theory, it can detect RI fluctuations at any spatial frequency whatsoever. Based on a 5% noise floor, $\tilde{\Sigma}$ detects scales from ~22 to 200–700 nm (exact values depend on sample structure and thickness). In an example involving mass-density distribution characteristic of biological cell nuclei, we suggest the level of chromatin organization, which can be quantified via $\tilde{\Sigma}$.

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Interferometric spectroscopy of scattered light quantifies the statistics of refractive-index (RI) distribution inside weakly scattering media using the spectral variance of their epi-illumination, bright-field microscope image ($\tilde{\Sigma}^2$). Its sensitivity to changes in deeply subdiffractional RI correlation lengths has been previously demonstrated [1]. However, as the RI correlation length is a cumulative statistic of all length scales (LS) present within a sample, a change in its value implies a global structural reorganization at all LS, including those larger than the diffraction limit of light. Thus, when quantifying the LS sensitivity of $\tilde{\Sigma}$ through a statistic of RI distribution, it remains unclear precisely which structural LS within the sample are detected. Here we investigate two aspects of the LS sensitivity of $\tilde{\Sigma}$. First, we determine whether there is a fundamental limit to the size of structures it can detect. Second, we identify which LS within complex samples with continuous random RI distribution have the largest contribution to its measured value. Furthermore, in an example of mass-density distribution characteristic of biological cell nuclei, we assess which LS of chromatin organization can be measured via $\tilde{\Sigma}$.

We model a far-zone, epi-illumination, bright-field microscope with a small numerical aperture of illumination and wavelength-resolved image acquisition. Our sample geometry includes an RI-matched substrate, an RI-mismatched superstrate (e.g., air), and a uniform thickness L, which is smaller than the microscope's depth of field (for most setups, 0.5–15 μm). We define the continuous, spatially varying RI of a weakly scattering sample as $n(\mathbf{r})$, and its fluctuating part normalized by the mean value (n_1) as $n_\Delta(\mathbf{r}) = (n(\mathbf{r}) - n_1)/n_1$. Using Born approximation to compute the scattered field inside the sample and ray optics to describe field propagation across its boundaries (theory developed in [1]), the expected value of spectral variance $\tilde{\Sigma}^2$ is found through the integral of the power spectral density (PSD, $\Phi_{\mathbf{n}_\Delta}$) of n_Δ within a frequency-space volume T_{3D}:

$$\tilde{\Sigma}^2 = \frac{\Gamma^2 k_c^2 L}{\Delta k} \int_{T_{3D}} \Phi_{\mathbf{n}_\Delta}(\mathbf{k}) \mathrm{d}^3\mathbf{k}, \tag{1}$$

where k_c is the central wavenumber of the instrument's bandwidth Δk (evaluated inside the sample) and Γ is composed of reflectance and transmission Fresnel coefficients, $\Gamma = 4n_0 n_1 (n_0 - n_1)/(n_0 + n_1)^3$, with n_0 being the RI of a sample's superstrate. T_{3D}, as described in detail in [1], includes spatial frequencies $\mathbf{k}$ with longitudinal coordinates k_z between $2k_1$ and $2k_2$ (k_1 and k_2 denoting the lower and upper wavenumbers of the instrument bandwidth), contained within a radial distance k_zNA from the k_z axis (NA denoting the numerical aperture of light collection). For consistency, in this Letter we consider 500–700 nm bandwidth, NA = 0.6, and n_1 corresponding to fixed biological media, $n_1 = 1.53$ [2,3].

We begin the LS sensitivity analysis of $\tilde{\Sigma}$ by identifying spatial RI fluctuations inside a sample at which frequencies it can detect. To isolate the sensitivity to a given spatial frequency of RI fluctuations, we numerically evaluate $\tilde{\Sigma}$ measured from a sample composed of periodic structures of that frequency only.

Using MATLAB (The MathWorks Inc.), we generate a zero-mean, statistically isotropic, random, 3D unbounded medium $n_\Delta^\infty(\mathbf{r})$, which is characterized by a single spatial frequency k_{LS} and variance σ_n^2 [Fig. 1(a)]. That is, the expectation of its PSD in spatial-frequency space is an infinitely thin spherical shell with a radius k_{LS} centered at the origin. Then we define the sample as a part of $n_\Delta^\infty(\mathbf{r})$ with thickness L:$n_\Delta(\mathbf{r}) = n_\Delta^\infty(\mathbf{r}) T_L$, where T_L is a windowing function along the z axis. It is important to note that the PSD of $n_\Delta(\mathbf{r})$, due to its finite thickness, is no longer a spherical shell and is expressed as $\Phi_{\mathbf{n}_\Delta} = |\sigma_n \delta(\mathbf{k} - k_{\mathrm{LS}}) \otimes \mathcal{F}\{T_L\}|^2$, where $\mathcal{F}$ denotes a unitary Fourier transform operator and $\otimes$ denotes convolution [Fig. 2(a)]. Finally, we obtain $\tilde{\Sigma}^2$ by computing the integral of $\Phi_{\mathbf{n}_\Delta}$ within T_{3D}. For comparison, to accentuate the effect of the finite sample thickness, we repeat the analysis omitting the convolution with $\mathcal{F}\{T_L\}$, which corresponds to bulk media with $L = \infty$.

Referring to Fig. 2(b), we establish that $\tilde{\Sigma}$ can sense RI fluctuations at any spatial frequency whatsoever and has an enhanced sensitivity to frequencies with $1/k_{\mathrm{LS}}$ between 20 and 40 nm ($1/2k_2 = 26$ nm and $1/2k_1 = 36$ nm). Due to the finite L, weakly scattering structures of any size and

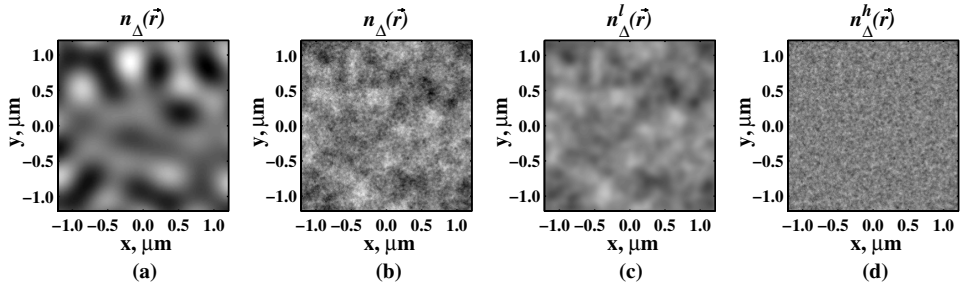

Fig. 1. (a) Cross section of a single spatial-frequency medium with n_1/k_{LS} of 100 nm. (b) Cross section of $n_\Delta(\mathbf{r})$ with an exponential SCF and the corresponding (c) $n^l_\Delta(\mathbf{r})$ and (d) $n^h_\Delta(\mathbf{r})$ for $W = 100$ nm.

shape, including RI fluctuations with subdiffractional frequencies above $2k_2$, can still be detected. This is explained by the fact that the Fourier transform of finite structures, due to the convolution with $\mathcal{F}\{T_L\}$, is nonzero inside T_{3D}. Accordingly, lower values of L correspond to wider $\mathcal{F}\{T_L\}$, resulting in higher sensitivity of $\tilde{\Sigma}$ to spatial frequencies outside T_{3D}.

We emphasize that T_{3D} includes values of $|\mathbf{k}|$ only below $2k_2$; hence the physics behind the fundamental limit of diffraction remain unbroken. Meanwhile, since the Fourier transform of RI fluctuations of a finite length with frequencies above $2k_2$ (as well as below $2k_1$) is nonzero inside T_{3D}, those fluctuations are detectable by $\tilde{\Sigma}$.

While $\tilde{\Sigma}$ is most sensitive to $1/k_{\mathrm{LS}}$ of 20–40 nm, it is not obvious from the experimental perspective which internal structures this LS range refers to. Moreover, for practical cases of samples containing multiple LS, a universal interval of LS having the largest contribution to the measured $\tilde{\Sigma}$ cannot exist, as it depends on the sample structure. First, it is simply a matter of which LS and in what proportion are present. Second, as shown above, the sensitivity of $\tilde{\Sigma}$ depends on L. And finally, owing to the nonlinear relation between $n_\Delta(\mathbf{r})$ and $\Phi_{\mathbf{n}_\Delta}$, contributions from different internal structures are not independent. Hence, the range of LS predominantly detected by $\tilde{\Sigma}$ can be found only for a specific structure. Below, we determine the LS sensitivity range for samples with various properties of internal organization, including analytically defined as well as experimentally obtained forms of RI spatial correlation function (SCF).

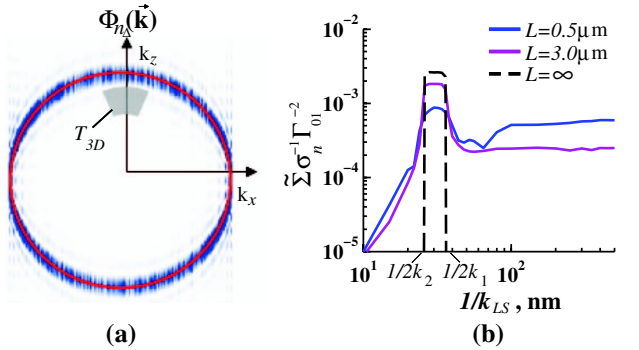

Fig. 2. (a) Cross section of PSD of $n^\infty_\Delta(\mathbf{r})$ (red) with $k_{\mathrm{LS}} > 2k_2$, and the PSD of the abridged sample $n_\Delta(\mathbf{r})$ (blue). (b) $\tilde{\Sigma}/\sigma_n\Gamma^2_{01}$ as a function of the spatial frequency of RI fluctuations for samples with different thicknesses (all wavenumbers evaluated inside the sample). Γ^2_{01} denotes $(n_0 - n_1)^2/(n_0 + n_1)^2$.

In order to identify whether a particular LS has a significant contribution to $\tilde{\Sigma}$, we "remove" it from the sample and calculate the consequent change in $\tilde{\Sigma}$ (for convenience, particle sizes rather than RI frequencies are measured, [4]). Thus, for lower LS analysis, we introduce a perturbed medium $n^l_\Delta(\mathbf{r})$, which represents the original $n(\mathbf{r})$ convolved with a 3D Gaussian filter $G(\mathbf{r})$ with FWHM W:$n^l_\Delta(\mathbf{r}) = (n^\infty_\Delta(\mathbf{r}) \otimes G(\mathbf{r}))T_L$, as a result of which LS lower than W are removed from $n(\mathbf{r})$. Similarly, $n^h_\Delta(\mathbf{r})$ represents $n(\mathbf{r})$ with high LS removed: $n^h_\Delta(\mathbf{r}) = n_\Delta(\mathbf{r}) - n^l_\Delta(\mathbf{r})$ [Figs. 1(b)–1(d)]. Using Eq. (1), we obtain the spectral variance of a microscope image that would be measured from a sample if all LS lower ($\tilde{\Sigma}^{l2}$) or higher ($\tilde{\Sigma}^{h2}$) than W were removed:

$$\tilde{\Sigma}^{l2} = \frac{\Gamma^2 k_c^2 L}{\Delta k}\int_{T_{3D}} |\mathcal{F}\{n^\infty_\Delta\}\mathcal{F}\{G\} \otimes \mathcal{F}\{T_L\}|^2 \mathrm{d}^3\mathbf{k},$$
$$\tilde{\Sigma}^{h2} = \frac{\Gamma^2 k_c^2 L}{\Delta k}\int_{T_{3D}} |\mathcal{F}\{n^\infty_\Delta\}[1 - \mathcal{F}\{G\}] \otimes \mathcal{F}\{T_L\}|^2 \mathrm{d}^3\mathbf{k}. \quad (2)$$

Since an analytical solution for $\tilde{\Sigma}^l$ and $\tilde{\Sigma}^h$ cannot be obtained, we evaluate Eq. (2) numerically. As before, we generate a zero-mean random 3D array with the desired form of SCF using MATLAB. Then we multiply the Fourier transform of the medium either by $\mathcal{F}\{G(\mathbf{r})\}$ or by $[1 - \mathcal{F}\{G(\mathbf{r})\}]$ (to remove short and long LS, respectively), after which we convolve it with $\mathcal{F}\{T_L\}$ to account for the finite thickness. We obtain $\tilde{\Sigma}^{l2}$ and $\tilde{\Sigma}^{h2}$ by computing the integral of the squared absolute value of the resultant array within T_{3D}.

Importantly, when a Gaussian particle described by W is contained inside $n^\infty_\Delta(\mathbf{r})$, only a fraction of that feature may be included in the truncated medium $n^\infty_\Delta(\mathbf{r})T_L$. Hence we define its effective size W_{eff} as W multiplied by the expected value of the particle fraction inside the longitudinal interval $(-L/2, L/2)$:$(-1 + e^{-C^2})/(C\sqrt{\pi}) +$ $\mathrm{Erf}(C)$, with Erf denoting the error function and $C = 2L\sqrt{\ln 2}/W$.

We first consider the sample to have an exponential SCF. We calculate the spectral variance measured after removing low ($\tilde{\Sigma}^l$) or high ($\tilde{\Sigma}^h$) LS relative to that measured from the original sample ($\tilde{\Sigma}$). Results of this LS perturbation analysis, shown in Fig. 3, lead to two important conclusions. First, from the region of the steepest decline in $\tilde{\Sigma}^h/\tilde{\Sigma}$ and $\tilde{\Sigma}^l/\tilde{\Sigma}$, we find that $\tilde{\Sigma}$ is most sensitive to LS below 200 nm. Second, we determine that the limited L most significantly affects the sensitivity of $\tilde{\Sigma}$ to large

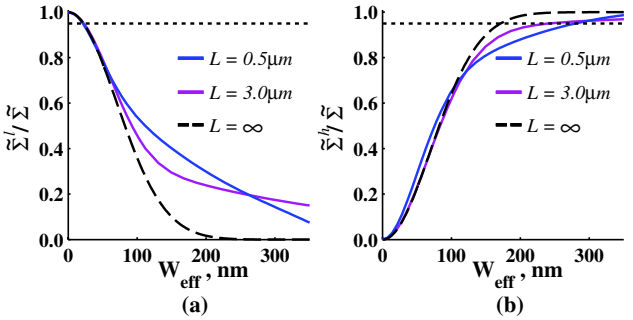

Fig. 3. Decrease in $\tilde{\Sigma}$ measured from samples with different thicknesses when (a) low and (b) high LS are perturbed. Data shown for RI correlation length of 100 nm. Dotted horizontal line indicates the threshold of a 5% change in $\tilde{\Sigma}$.

structural LS. This results in a qualitative difference in the behavior of $\tilde{\Sigma}^l$ and $\tilde{\Sigma}^h$ calculated for different values of L: whereas for $L = \infty$ they saturate at $W_{\text{eff}} > 200$ nm, for thin samples they remain slowly varying functions of W_{eff}. This is in agreement with the single-k_{LS} analysis, which established that smaller L expands the range of detected LS.

Whereas we have shown that theoretically $\tilde{\Sigma}$ can sense all LS, in experiments its sensitivity is limited by the signal-to-noise ratio, which varies largely depending on the instrumentation and scattering power of the studied sample. Thus, to give an estimate of the LS sensitivity of $\tilde{\Sigma}$, we define it as the range of W_{eff} for which perturbing the corresponding higher or lower LS within the sample causes at least a 5% change in $\tilde{\Sigma}$. That is, the lower (r_{min}) and upper (r_{max}) limits of LS sensitivity are the values of W_{eff} such that $\tilde{\Sigma}^l(W_{\text{eff}} = r_{\text{min}}) = 0.95\tilde{\Sigma}$ and $\tilde{\Sigma}^h(W_{\text{eff}} = r_{\text{max}}) = 0.95\tilde{\Sigma}$. Applying this threshold, we find the range of LS detected by $\tilde{\Sigma}$ to be 22–240 nm for $L = 0.5$ μm, 23–281 nm for $L = 3$ μm, and 22–171 nm for $L = \infty$. For comparison, thresholds of a 1% and a 25% change in $\tilde{\Sigma}$ would define corresponding LS sensitivity ranges of 9–360 nm and 56–124 nm for $L = 0.5$ μm, 10–360 nm and 56–124 nm for $L = 3$ μm, and 10–212 and 53–116 nm for $L = \infty$.

Naturally, since the range of LS detected by $\tilde{\Sigma}$ depends on the LS composition of the sample, r_{min} and r_{max} change as a function of RI correlation length l_c (Fig. 4). While there is no analytical relationship describing r_{min} and r_{max} in terms of l_c and L, their qualitative behavior is as follows. As l_c increases, the amount of large LS

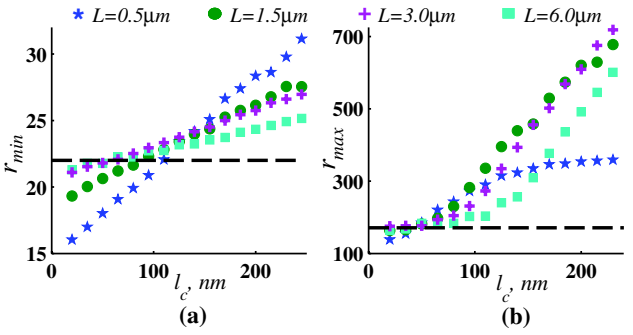

Fig. 4. Dependence of (a) r_{min} and (b) r_{max} corresponding to the 5% threshold on L and l_c. Black dashed lines indicate $r_{\text{min}} = 22$ nm and $r_{\text{max}} = 171$ nm corresponding to $L = \infty$.

increases, and hence the range of detected LS shifts upward, increasing r_{min} and r_{max} (note that r_{max} cannot reach L and therefore saturates below it at $L/l_c \leq 3$). Expectedly, the effect of a finite L on r_{max} is much greater in magnitude than that on r_{min}. In contrast, at small l_c the sensitivity of $\tilde{\Sigma}$ to shorter LS is emphasized, which is quantified by values of r_{min} lower than that for $L = \infty$ and by r_{max} approaching its value corresponding to $L = \infty$ when $L/lc \gg 3$.

We next employ the present LS perturbation analysis to determine which structures inside biological cells are quantified by the spectral content of their microscope image. We model RI distribution typical to biological cell nuclei using mass-density SCF measured from transmission electron microscopy (TEM) images reported in [5]. Following standard TEM protocol, human colonic cell nuclei were stained with specific to DNA osmium tetroxide, sectioned, and imaged. The gray-scale image intensity, assumed to be proportional to the local density of chromatin, was used to compute the mass-density SCFs of 36 micrographs, after which an average SCF of nuclear material was obtained [5]. Since the RI of biological media is a linear function of mass density [2], the SCF of RI distribution equals that of mass-density distribution with a constant prefactor. The prefactors of mass-density SCF are unknown (due to the variability in the depth of staining), and therefore we normalize the RI SCF to 1 at the origin ($r = 0$).

We use the information contained in the experimental SCF by following two approaches. First, we fit an exponential SCF to the experimental (r^2 value of the fit between 39 and 1000 nm is 0.99) and find the exponential correlation length l_c to be 156 nm. We then follow the previously described analysis for two values of L, 1.5, and 6.0 μm (mimicking the thickness of squamous and columnar epithelial cell nuclei). Second, to evaluate how well the model of an exponentially correlated medium applies to biological cells, we use the experimental SCF directly. Since the experimental SCF is only defined at $r \geq 39$ nm (resolution of TEM micrographs), we extend it to $r = 0$ using the fitted exponential SCF at $r < 39$ nm. We then generate random media with SCF equal to the experimental and perform LS sensitivity analysis.

An excellent match between $\tilde{\Sigma}^l/\tilde{\Sigma}$ and $\tilde{\Sigma}^h/\tilde{\Sigma}$ calculated based on analytical and experimental SCFs is obtained for all values of W_{eff} (Fig. 5). Applying the 5% threshold, for $L = 1.5$ μm we find that $\tilde{\Sigma}$ detects intranuclear structures from 25 to 427 ± 11 nm based on the analytical SCF, and from 25 to 441 ± 11 nm based on the experimental (uncertainty intervals correspond to the standard error between 20 samples; error is not shown when it is <0.5 nm). For $L = 6$ μm, we find that $\tilde{\Sigma}$ detects structures from 23 to 324 ± 5 nm (for analytical SCF) and from 23 to 334 ± 5 nm (for experimental). Additionally, from $\tilde{\Sigma}^l$ and $\tilde{\Sigma}^h$ calculated for both thicknesses and types of SCFs, we note that the largest contribution to $\tilde{\Sigma}$ measured from biological cell nuclei comes from structures smaller than 200 nm in size (Fig. 5).

To summarize, we establish that the interferometric spectroscopy of scattered light has a unique ability to detect spatial RI fluctuations of any frequency inside a thin, weakly scattering sample. Most importantly, its

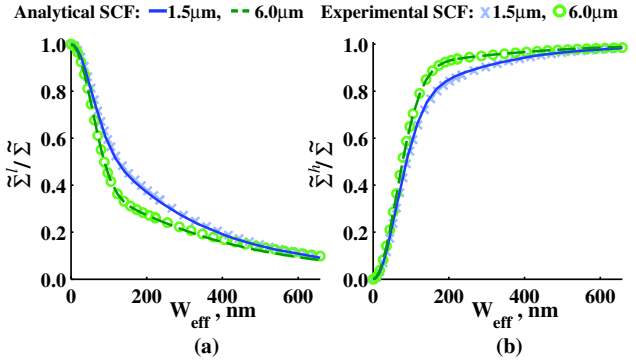

Fig. 5. Relative change in $\tilde{\Sigma}$ when (a) lower ($\tilde{\Sigma}^l/\tilde{\Sigma}$) and (b) higher ($\tilde{\Sigma}^h/\tilde{\Sigma}$) LS are perturbed. Calculation performed for samples with SCF, which is experimentally measured from TEM images (blue markers for samples with thickness 1.5 μm and green for 6.0 μm) and analytically defined as exponential (blue solid line for $L = 1.5$ and green-dashed for $L = 6.0$ μm).

sensitivity to subdiffractional spatial frequencies (above $2k_2$) is limited not by the diffraction of light but by the signal-to-noise ratio of the detector.

Based on a 5% noise floor, we report the range of LS $\tilde{\Sigma}$ detects inside complex media with an exponential SCF. The smallest detectable LS is determined to be $\sim\lambda/26$ (22 nm) for all tested values af L and l_c. The sensitivity to large LS, in turn, is found to be highly dependent on L. As a result, the largest detectable LS is $\sim\lambda/3$ (200 nm) for samples with short RI correlation lengths ($l_c < 100$ nm) and reaches values as high as $\sim\lambda$ for $l_c > 100$ nm. Precise sensitivity limits for a wide range of sample parameters relevant to the case of isolated biological cells are reported.

We emphasize that the present analysis does not address the sensitivity of $\tilde{\Sigma}$ to material rearrangement processes such as macromolecular aggregation or decondensation. Mass-preserving structural rearrangement is characterized by alterations in the shape of PSD and a constant total power of RI fluctuations. Thus the sensitivity of $\tilde{\Sigma}$ to such processes is best evaluated through its functional dependence on parameters of the shape of PSD, which is analogous to that reported in [1].

Finally, we substantiate the relevance of the presented analysis and its conclusions to biological media, which are not necessarily quantified by an exponential SCF. We establish that spectral variance of an epi-illumination, bright-field microscope image, utilized for the quantification of subdiffractional intracellular structures in techniques such as partial wave spectroscopic microscopy [6], measures LS between 25 and 400 nm, which, in terms of nuclear organization, correspond to structures from a chromatin fiber to nucleoli and chromatin aggregates barely resolvable by a conventional microscope. Accordingly, we conclude that the largest contribution to $\tilde{\Sigma}$ measured from biological cell nuclei must come from structures smaller than 200 nm in size. Large LS such as nuclear size or shape would have virtually no contribution to the monitored signal.

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