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COMMENT

How a phase image of a cell with nucleus refractive index smaller than that of the cytoplasm should look like? A Comment on two papers by Steelman et al. and Schürmann et al.

Read the Responses to this Comment: e201800091 and e201800095

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In recent papers Steelman et al. ("Is the nuclear refractive index lower than cytoplasm? Validation of phase measurements and implications for light scattering technologies") and Schürmann et al. ("Cell nuclei have lower refractive index and mass density than cytoplasm") obtained quantitative phase images of whole cells of various types and corresponding isolated nuclei and concluded that the refractive index (RI) of the nucleus is significantly smaller than that of the cytoplasm. The comment shows that this conclusion and assumptions used in retrieving the RI necessarily imply a characteristic dip in the center of the whole-cell phase images. This dip is not present in any of the phase images in the discussed papers, which is a strong argument against the conclusion of smaller nucleus RI. It is also discussed whether a different processing of the phase images can help to clarify this issue.

KEYWORDS
light scattering, microscopy, nucleus, phase imaging, refractive index

Let us, first, recall the workflow used in the discussed papers [1, 2]. The raw measured images were processed by filtering, phase extraction and unwrapping, and background removal to produce a (clean) phase image [3]. Then a circle was fitted to the cell (or isolated nucleus) edge to determine its center and radius R and the simple-transmission approximation was used to relate the phase φ and RI n:

$$\varphi(r)=\frac{4\pi}{\lambda}(n(r)-n_0)\sqrt{R^2-r^2},\ r\le R, \tag{1}$$

where n_0 is the RI of the host medium, λ is the wavelength in the vacuum, r is distance from the corresponding pixel to the circle center. Although Schürmann et al. [2] acknowledge the diffraction-limited resolution of the phase images to be 850 nm (at λ = 633 nm), the above processing implicitly assumes that the diffraction (scattering or reflection) may only blur the RI maps, but does not affect the RI averaged over the whole cell. We will discuss this assumption later in this comment, important for now is that it was assessed (even if partly) only for homogeneous spheres.

The RI map obtained using Equation (1) was further averaged (weighted by height) to produce a single RI value. Importantly, the inhomogeneity of the cell is not accounted for during processing, but only used afterward (equation (1) of [1]) to estimate the RI of the cytoplasm n_{cyt} from the obtained average RIs of the whole cell n_{cell} and isolated nucleus n_{nuc}:

$$n_{cyt}=\frac{n_{cell}d_{cell}^3-n_{nuc}d_{nuc}^3}{d_{cell}^3-d_{nuc}^3}, \tag{2}$$

where d_{cell} and d_{nuc} are diameters of the cell and nucleus, respectively. Let us now accept the RI values deduced in refs. [1, 2] and simulate the whole-cell phase images assuming simple transmission of the incident wave.

First, let us consider a MCF-7 cell from figure 2 (right) of ref. [1]. The values of n_0 = 1.335, d_{nuc} = 9 μm, and d_{cell} = 13 μm are taken from section 2.3 of [1]; the ratio of the diameters is similar to that in the phase image. The values of λ = 540 nm and average over population n_{cell} = 1.378

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and $n_{nuc} = 1.351$ are taken from section 3.1 of [1], implying $n_{cyt} = 1.391$ with the help of Equation (2). Note, that Figure 1 (top-left) of [1] implies a different average $n_{nuc} = 1.348$, but that would only make the following dip feature more pronounced. Unfortunately, the specific wavelength corresponding to "Normal" phase image on Figure 2 (right) of [1] has not been specified, but λ affects only the overall magnitude of phase images given by Equation (1). Generalizing Equation (1) to a concentric coated sphere results in.

$$\varphi = \frac{4\pi}{\lambda}\left(n_{cyt} - n_0\right) \times \begin{cases} \sqrt{R_{cell}^2 - r^2} + \dfrac{n_{nuc} - n_{cyt}}{n_{cyt} - n_0}\sqrt{R_{nuc}^2 - r^2}, r \le R_{nuc}, \\ \sqrt{R_{cell}^2 - r^2}, \quad R_{nuc} < r \le R_{cell}, \end{cases} \tag{3}$$

where the ratio $\frac{n_{nuc} - n_{cyt}}{n_{cyt} - n_0}$ is a negative number with magnitude not much smaller than 1, whenever the nucleus is significantly less dense than the cytoplasm (in the considered case this ratio equals −0.72). The simulated φ along the diameter is shown in Figure 1A—it shows a dip in the center or, alternatively, a large-phase ring with radius of about 4.5 μm. None of this is present in Figure 2 (right) of [1]. Importantly, those characteristic features are not expected to vanish due to the diffraction blurring or shift of the nucleus inside the cell. In Figure 1B we show the apparent RI, obtained by solving Equation (1) for $n(r)$. It manifests even more pronounced dip than the phase, but, unfortunately, ref. [1] does not contain a RI map for direct comparison.

Second, let us consider a HL60 cell, which phase images at $\lambda = 633$ nm are shown in Figures 2(a), S1(c) and

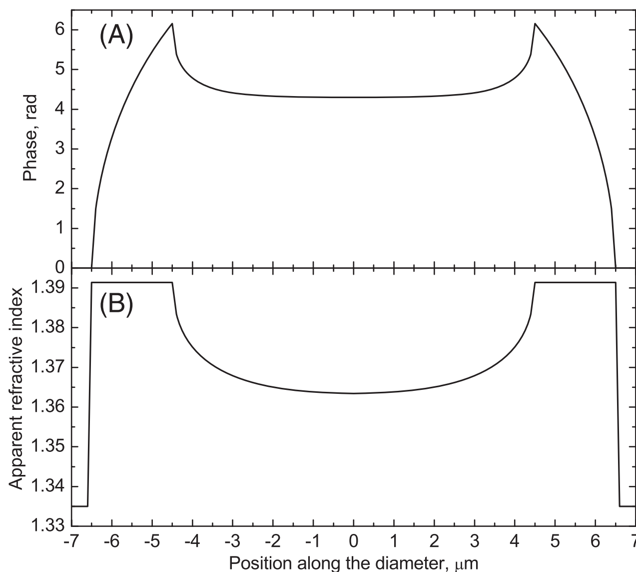

FIGURE 1 Simulated phase shift (A) and apparent refractive index (B) for MCF-7 cell from [1] using a concentric coated sphere model and the simple-transmission approximation

S2(a,b) of ref. [2]. The values of $n_0 = 1.335$, $n_{cell} = 1.378$ and $d_{cell} \approx 13$ μm are taken from section 3.1 and Figure S2(c) of [2], respectively. With respect to the nucleus, Schürmann et al. noticed its significant shrinking during isolation (average volume from 606 to 450 μm^3, see Figure 3(a,b) of ref. [2]) and used $n_{nuc}^{is} = 1.359$ of isolated nucleus to estimate $n_{nuc} = 1.348$ assuming the constant dry mass (Section 3.6 of [2]). In the following, both these cases are considered; the corresponding diameters $d_{nuc}^{is} = 4.75$ μm and $d_{nuc} = 5.25$ μm are obtained directly from the nucleus volumes. The simulated phase images and RI maps are shown in Figure 2, depicting only a half the symmetric profiles to avoid redundancy.

Figure 2A contradicts the actually measured profile (Figure S2(b) of [2]) in 2 respects: the latter has no dip and is almost constant over most of the internal area (even for $r > R_{nuc}$)—none of this can be explained by diffraction blurring, since the corresponding features are several micrometer wide. In Figure 2C both these contradicting features are present, but at a lesser scale. However, even more striking difference can be seen between the simulated and measured RI maps—compare Figure 2 (c,d) with Figure S1(c) of ref. [2]. Again, the latter contains no sign of a dip.

Let us further discuss the implications of the presented counterexamples. They do not imply that the RI of the nucleus is larger than that of the cytoplasm; instead they prove that simple-transmission approximation is self-contradictory when applied for processing of real phase images of inhomogeneous cells. In other words, both Steelman et al. [1] and Schürmann et al. [2] correctly deduced the cell parameters under this approximation. However, a simple next step discussed in this comment brings it into a contradiction with the original phase image. One may think that the simple-transmission approximation is fine up to the diffraction blurring of the final RI maps—the above counterexamples show that this is not true. A crude estimate of the magnitude of the problem in terms of RI can be obtained by the scale of the characteristic dips—between 0.01 and 0.02. However, this estimate is only a speculation warranting further study.

The only potential possibility to resolve this issue is to bring the discussion from ad hoc approximation into the realm of the rigorous light scattering (frequency-domain electromagnetics) [4]. Schürmann et al. [2] actually used the

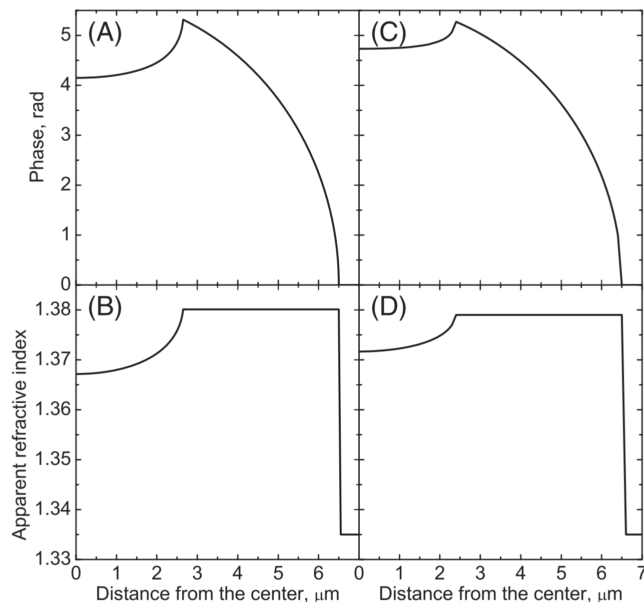

FIGURE 2 Simulated phase shift (A, C) and apparent refractive index (B, D) for HL60 cell from ref. [2] using a concentric coated sphere model and the simple-transmission approximation. Right (C, D) and left (A, B) parts correspond to the parameters measured directly for the isolated nucleus and those deduced for the nucleus inside the cell, respectively

rigorous Mie theory to simulate the phase images of homogeneous spheres and showed that the standard processing under the simple-transmission approximation leads to satisfactory accuracy (within 0.005 in RI, see Figure S7 of ref. [2]). The same conclusion is supported by Figure S4(a) of ref. [2], depicting phase images of hydrogel beads, which look qualitatively similar to that expected from Equation (1). Steelman et al. (section S2 of ref. [1]) analyzed another aspect of the scattering by a sphere—the energy in reflected and transmitted rays, but it only raises more questions. First, it is based on geometrical optics and, thus, ignores diffraction altogether (which can be significant even for low RI contrast). Second, the transmitted ray is deflected by a nonnegligible angle after passing 2 nonparallel interfaces. Third, small difference in energy does not necessarily imply small difference in phase.

Importantly, none of the above tests have direct implications for the inhomogeneous cells. The characteristic width of the cytoplasm is $(d_{\text{cell}} - d_{\text{nuc}})/2$, which is usually significantly smaller than d_{nuc} (e.g., Figure 1), implying larger effect of diffraction. This can be studied by simulating the rigorous phase image of the concentric coated sphere (based on the Mie theory and specific collection optics) and testing the processing algorithm on it. One may even try to fit the measured phase image with theoretical ones for the coated-sphere model, similar to what has been done for holograms [5]. Another issue is that cells have granules and distortions of the cell or nucleus membranes on the scale comparable to the wavelength. It is not clear a priori how important are such inhomogeneities for a phase image of a specific cell. But, if it cannot be neglected, the quantitative retrieval of RI of cell components from the phase image seems a very complex problem.

To conclude, Steelman et al. [1] and Schürmann et al. [2] presented evidences suggesting that the common assumption that the nucleus RI is larger than that of the cytoplasm is not true for several cell types. However, this comment shows that their processing of the phase images is also questionable, so the opposite conclusion about RI for the discussed cells is not necessarily true as well. At least, the specific values of RI of the nucleus inside the cell may be significantly different from the reported values. Further research, including more complicated processing (inversion) of the phase images, is required to clarify this issue.

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