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The authors declare no conflict of interest.

Significance

Accurate measurement of cell size is critical in studies of cell growth. Optical methods based on interferometry are known to be suitable for attached cells, but the existing techniques were originally designed for thin samples and are not ideal for thick samples, such as mitotic cells. Synthetic phase microscopy (SPM), a new tomographic interferometric method, offers an elegant solution to this problem. This paper demonstrates the ability of SPM to measure the growth of mammalian cells accurately, and it demonstrates a clear requirement for feedback in the growth process.

Supporting information online

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incident onto a cell is diffracted from the cell boundary and cell’s internal organelles, and thus propagates within the sample like the ripples in water rather than a straight ray. Thus, the scalar diffraction theory may be more appropriate for interpreting the scattered light field from biological samples (20).

Here, we report a rigorous treatment of the light scattering by heterogeneous 3D structures in a cell to measure the dry mass in living cells accurately, regardless of their shape or optical depth. Our method measures the dry mass of thick samples as accurately as for thin samples; therefore, we can use it to study both size variation upon division and the growth kinetics of living cells. Specifically, we measure the dry mass of daughter cells immediately after division in L1210 lymphoblastoid cells and two epithelial cell types, HT-29 and RKO human colon cancer cells. We also measure the mass distribution of these cells growing naturally in constant conditions. Then, using the Collins–Richmond conservation principle (21), we obtain the relationship between growth rate and cell mass. Our findings of the asymmetry of cell division and the mass dependence of growth in epithelial cells demand an intrinsic mechanism for growth control like that proposed for bacteria (22), yeast (3, 4) erythroblasts (23), and lymphoblasts (7, 10).

Results

Synthetic Phase Microscopy for Dry Mass Measurement.

The light field scattered from a sample carries the information about the sample in the form of amplitude modulation, wavefront distortion, or both. The wavefront modulation or phase delay, which can simply be related to the sample’s refractive index in the visible regime, can be measured by Shack–Hartmann sensors (24, 25), interferometry (26–29), or propagation-based methods (30, 31). The linear relationship between the refractive index and the concentration of organic molecules is established well (32–35). Notably, for most of a cell’s chemical components, the specific refraction increment α relating the refractive index and mass concentration remains constant regardless of the chemical identity of the biomaterial (35). For instance, the mean proportionality coefficient for the entire set of human proteins is 0.190 mL/g, with an SD of only 0.003 mL/g (35).

The scalar diffraction theory predicts that the scattered field for a specific angle of illumination onto an object provides a portion of the object spectrum in 3D spatial frequency space (36). Importantly, the information needed to calculate the dry mass of a specimen is mostly contained in the W = 0 plane (SI Materials and Methods), where W is the spatial frequency coordinate corresponding to the optical axis Z (Fig. 1A). Under the Rytov approximation (37), which is valid for most biological samples, the following relationship holds in the Fourier plane between the areal dry mass density σ(X, Y) and the sample-induced phase delay \( \Phi_{o}(X, Y) \) recorded for a specific illumination angle (SI Materials and Methods):

\[
\sigma(X, Y) = \int_{\Omega} \tilde{\sigma}(U, V) e^{i2\pi(UX + VY)} dU dV,
\]

where \( \Omega \) is the spatial frequency support determined by the N.A. of a synthetic phase microscopy (SPM) system. We note that the areal density image obtained with SPM (Fig. 1D) is free from the diffraction artifact that will be described more extensively below. In addition, the areal density in Eq. 2 is equivalent to the numerical projection of the 3D refractive index map obtained with optical diffraction tomography (ODT) (38). Therefore, SPM provides transverse resolution similar to that obtained with ODT, which is double the resolution available from single-shot phase imaging. We note that the computational efficiency is much higher in SPM, because it maps the measured scattered fields onto a 2D plane rather than a 3D spatial frequency space as in ODT. Fig. 1D compares areal density images of an RKO human colon cancer cell obtained with SPM and single-shot phase imaging. It is clear that the noise level in SPM is significantly lower than in single-shot phase imaging. For example, the dry mass value within the dotted region in the SPM image amounts to only 2.5 fg, which is comparable to the measurement noise of 6 fg in a suspended microchannel resonator (7). The high measurement sensitivity of SPM is attributed to significantly reduced coherent speckles, as in partially coherent imaging (39). To test the measurement accuracy of SPM, we quantified the refractive index of polystyrene beads and compared it with the value measured using a refractometer for bulk material (40). Note that we obtained the 2D phase images with SPM and calculated the refractive index of the beads using the spherical shape of the beads. The mean refractive index for the 20-μm polystyrene beads (n = 44) was measured to be 1.5857 with an SD of 2 × 10^{-4}. This value matches very well with the refractive index of bulk polystyrene, which is 1.5872 at 633 nm. Fig. 2A shows how the diffraction from small organelles can affect measured phase, and thus dry mass value. Phase images (i) and (ii) were obtained with single-shot phase imaging, whereas phase images (iii) and (iv) were acquired with the SPM method, and thus are diffraction-corrected. The objective focus was placed at the center of the bead for phase images (i) and (iii), and at 2 μm above the center for phase images (ii) and (iv). If the projection assumption were valid or its error negligible, phase images (i) and (ii) should have shown a similar phase delay pattern even though they are taken at different heights; this is clearly not the case. This observation is consistent with our previous result that out-of-focus features are blurred and generate diffraction artifacts when the projection approach is adopted in tomographic reconstruction (38). The cell has a multitude of organelles, such as the nucleolus, mitochondria, and lipid droplets, and the objective focus is usually fixed at a location minimizing the diffraction pattern from the cell boundary. Therefore, out-of-focus organelles will introduce artifacts in a manner similar to the out-of-focus polystyrene bead in phase image (ii). On the other hand, SPM can correct this artifact; the phase delay patterns in phase images (iii) and (iv) are quite similar, although not exactly the same, perhaps due to the Brownian motion of the bead during data acquisition. Next, we tested the robustness of SPM for measuring cells of different shapes and optical thicknesses. We grew RKO cells on a polyl-lysine–coated coverslip for 48 h, removed the slide from the incubator, and washed the cells with PBS. Using an on-stage flow chamber with an embedded heater, we observed the change in the measurement of cells’ dry mass after adding trypsin. Within 10 min, the three cells in the middle of Fig. 2B changed their shape and their optical thickness at the center increased about 60%. However, the change in the dry mass measurement of the cells was less than 1% (SD) of the initial measurement. This shows that SPM measurements are almost entirely insensitive to cell thickness. In SPM, the cell dry mass is calculated by integrating
the measured areal density over the cell area. To determine the cell area, we use thresholding, which identifies the cells in the culture medium, together with manual selection, to separate one cell from another. Using two threshold levels (0.1 and 0.2 pg/μm²) and repeating the selection of cell boundary three times, we estimated the uncertainty of SPM mass measurement due to boundary selection at less than 1 pg in cells of an average mass of 348 pg, that is 0.3% (n = 18).

Cell Division Symmetry and Size Dependency of Growth Rate. Careful analysis of nearly spherical L1210 lymphoblasts revealed up to 7% differences in volume among daughter cells (10), emphasizing the need for a mechanism that regulates size homeostasis in these cells. However, optical measurements of adherent cells revealed volume changes in mitosis unrelated to biomass changes (41, 42), suggesting that cell size may not be accurately represented by volume at that stage. Therefore, it is not known to what extent biomass division is asymmetrical in animal cells, particularly for adherent amorphous cells. Dividing cells become round but not necessarily spherical, especially in adherent cell types. The asphericity (10), loss of contact area (9), or inadequacy of the projection model applied to L1210 lymphoblasts, which are round cells of epithelial origin from the colon, HT-29 (Fig. 4B) and RKO (Fig. 3B) were significantly greater than for L1210 cells.

Cells proliferating in an unchanged environment (steady-state population) maintain a time-invariant cell size distribution (i.e., although cell number increases with time, the probability density of the cell size distribution remains constant at any time). If the growth of cells is independent of their size, they could maintain the time-invariant size distribution independent of size checkpoint even if division is asymmetrical. By applying the Collins–Richmond model (21) to lymphoblastoid cell lines, Tzur et al. (10) showed a size-dependent growth rate and exponential growth pattern for these cells. Using our newly developed measurements, this analysis can now be improved. With respect to cell growth, mass is clearly a more relevant metric than volume. Moreover, this model for measuring growth patterns in adherent cells previously relied on assumptions with unknown validity (43). In this model, the growth rate of cells is calculated from three probability density functions for the asynchronous or steady-state populations, newly divided cells (newborns), and mitotic cells:

\[ n(m) = \gamma \left( 2 \int_0^m \phi(m')dm' - \int_0^m \theta(m')dm' - \int_0^m \chi(m')dm' \right) / \chi(m) \]

in which the functions \( \phi(m), \theta(m) \) are the probability densities in the size of newly divided cells (newborns) and mitotic cell populations, respectively, which, together, constitute the flux of division. The function \( \chi(m) \) is the probability density in the size of asynchronous cells. The parameter \( \gamma \) is the division rate, representing the rate of increase in population (cell number) with time [more details and derivation are provided in refs. 10, 21].

Fig. 4A–C shows the calculated result for the Collins–Richmond model applied to L1210 lymphoblasts, which are round and only slightly adherent (Fig. 4A), and two adenocarcinoma cells of epithelial origin from the colon, HT-29 (Fig. 4B) and RKO (Fig. 4C), which are asymmetrical and strongly adherent. The inputs for the model (Fig. S1) were calculated by kernel density estimation, a nonparametric way of estimating the probability density based on a finite dataset (44, 45). The thick lines in Fig. 4A–C show the average growth rate, and the colored...
areas represent the SD calculated by the propagation of errors method (10). We found the growth rate to be size-dependent for all three cell types. Together, results in Fig. 3 and 4 show that growth rates of proliferating lymphoblastoid and epithelial cells increase with cell mass and that the biomass of a dividing cell is distributed unevenly among daughters. These data are in accord with an exponential model of cell growth and imply the existence of an intrinsic mechanism that actively coordinates growth with division in epithelial cells.

**Discussion**

Cell growth, balanced by cell division, determines cell size, a fundamental parameter of cells. “Cell growth” is defined as an increment in biomass over time; however, size has a more complex definition. Geometric size [i.e., volume, length (in the case of symmetrical specimens)] is commonly used as a measure of size, and cell growth is typically expressed as volume increase over time. This preference for geometric volume is largely due to the convenience, accuracy, and availability of the Coulter counter, a readily available and accurate means of determining cell volume. Therefore, conclusions on the mechanism of cell size control are usually based on geometric size. Until recently, the best-supported conclusions have been in fission yeast, where “geometric size” appears to be sensed by intrinsic mechanisms in a way that coordinates cell growth with cell division (3, 4). Recent data showing exponential cell growth in suspension mammalian cells agree with this suggestion (7, 10).

Optical measurements of geometric size are relatively straightforward in symmetrical and rigid specimens, such as fission yeast, the most established models for size homeostasis research. Metazoan cells, particularly adherent ones, are generally amorphous and flexible, and are thus considerably more challenging for size measurements. At present, optical methods for volume measurement are inadequate for unambiguously deriving the growth curve of proliferating adherent cells. Confocal laser scanning microscopy (CLSM) can be used to map the cell border, but volume reconstruction using CLSM typically requires multilayer scanning and intensive illumination, putting the cells at a real risk of phototoxicity followed by abnormal growth, checkpoint arrest, and programmed cell death (46). In contrast, interferometry-based techniques use low-intensity transillumination and do not require any chemical staining or protein fluorescence labeling to measure the cell’s size or growth. Moreover, such methodology measures the cell’s biomass rather than volume or other mass surrogates.

Interferometric methods have been used for dry mass measurements for half a century, but it is only recently that these methodologies have reached the accuracy required for studying cell growth. These improvements in accuracy were made by improving noise suppression by adopting a common path configuration (19), by using broadband light (8), or by adding up multiple phase images (47). In this paper, we demonstrated a new method that is not only low in noise but corrects diffraction artifacts. This newly developed method (SPM) is based on a unique 2D data mapping of the scattered fields recorded at multiple angles of illumination. SPM efficiently provides an accurate value of cell dry mass unaffected by diffraction artifacts, and thus can be used for both thick and thin cells. In addition, as evidenced by the trypsin experiment, it has an estimated measurement precision of 1% of the total mass and even higher estimated measurement sensitivity (2.5 fg). Furthermore, SPM provides about twice as high spatial resolution as single-shot phase imaging.

With this improved method, we measured the dry mass of newborn and mitotic cells, as well as the mass distribution of...
Fig. 4. Size dependency of cell growth. Average cell growth rate vs. cell dry mass calculated for L1210 (A), HT-29 (B), and RKO (C) cells using the Collins–Richmond conservation equation (details are provided in main text). (D) Three growth rate vs. cell dry mass curves (L1210, HT-29, and RKO cells) and one growth rate vs. cell volume curve (L1210 cells from the study by Tzur et al. [10]) were normalized by their own critical size and the maximum growth rate.

asynchronous cells growing naturally. Using these parameters and the Collins–Richmond conservation equation, we calculated the mass dependency of the growth rate for L1210 lymphoblasts and two epithelial cell types, HT-29 and RKO. For L1210 lymphoblasts, we showed a roughly linear relationship between the average growth rate and dry mass; however, this dependency is not completely uniform over the size range, and presumably over the cell cycle. For most of the size range, the growth rate increases with increasing mass in a steady-state population; however, beyond a critical size (cell mass = 275 pg), this trend is reversed and growth rate declines as a function of mass. In 80% of the cells, however, division occurs before the cells reach this mass (Fig. 4A). The overall relationship between dry mass and growth rate we found was almost identical to that previously reported using volume as a measure of size (10). In fact, if normalized for the maximum, the plots are nearly overlapping, as shown in Fig. 4D, suggesting that the density of nonaqueous materials in the cell remains constant in the population at 135.5 g/L. Because cell density seems constant in proliferating L1210 cells (17), these findings establish our technology as a high-resolution tool for size and growth measurements in living cells.

Our previous work on lymphoblastoid cells helped resolve a long-standing debate of whether mammalian cells follow a linear or exponential growth pattern (10), and therefore whether there is a feedback control maintaining cell size. However, cells grown in suspension may be exceptional. Conlon and Raff (5) raised this issue in their paper on linear growth in Schwann cells, writing “Our findings also do not exclude the possibility that animal cells such as lymphocytes, which can proliferate in suspension like yeast cells, might use cell-size checkpoints to coordinate their growth with cell-cycle progression.” Applying the SPM method described here to two epithelial cell lines, HT-29 and RKO, we show in both cases that these cell lines, like L1210 cells, maintain a linear relationship between the average growth rate and cell mass over the majority of the size range. The Collins–Richmond plots calculated for both cell types were remarkably similar to that of L1210 (Fig. 4D) and demonstrate that the exponential growth model is not restricted to lymphoblastoid cells grown in suspension. In addition, we quantify with unprecedented accuracy the biomass distribution in daughter cells immediately after division. The average mass asymmetry was lowest in daughter L1210 cells (7.5%), recapitulating the 7% asymmetry estimated by volume (10). Biomass asymmetry was significantly greater in both epithelial cell lines, averaging ≥10% in both cases. Perhaps physical stress caused by surrounding cells, the uneven microenvironment a dividing adherent cell experiences, and the surface itself introduce biophysical perturbations that increase asymmetry relative to cells that are dividing freely in suspension. Partitioning ribosomes, total proteins, carbohydrates, and lipids with over 10% asymmetry is significant. In a simple exponential model of growth, where size is the only parameter affecting growth rate, daughter cells that divide unevenly will grow at a different pace. Thus, without a size gate that feeds back on the timing of division, cell size distribution in steady-state populations cannot remain constant. Fig. S2 illustrates this point; for example, if cells divide with 10% asymmetry and daughter cells grow without the size gate up to twice their original mass, the relative difference between the largest and smallest cells in the population, starting from a single cell, will be 92% after nine cycles. Therefore, in at least two adherent cell lines of mammalian origin, similar to the situation for unattached lymphoblast cells, there must be a cell-autonomous size regulator that couples cell growth to the cell cycle.

These results differ from those of Conlon and Raff (5) on adherent Schwann cell cultures, where a size-independent of growth was suggested. This might simply reflect differences in cell type or, alternatively, differences in experimental design. Interestingly, we found for all three cell types that large cells above a critical size (L1210: 271 pg, HT-29: 370 pg, and RKO: 275 pg) reverse their growth pattern. For all cell types, at least half of the cells (L1210: 80%, HT-29: 62%, and RKO: 56%) divide before reaching this critical size (Fig. S1 A, D, and G) (i.e., maintain a linear and monotonic relationship between mass and growth rate throughout their life cycle). For cells beyond this point, size seems to convey a slight growth inhibition. This growth limitation can be either “passive” (e.g., induced by biophysical properties like surface to volume ratio) or, alternatively, “active” (e.g., diluting a rate-limiting component below a critical value). In L1210 cells, the probability of cell division varies independently with cell size and cell age (10). It is therefore plausible that the growth of large cells is a bit delayed until “time” dominates and induces division. Such a mechanism can potentially limit cell size range in a steady-state population.

In summary, the approach we described here, along with others under development (7–10), enables the ability to measure growth in both suspension and attached cells under a wide variety of commonly studied conditions.

Materials and Methods

Microscope Setup. The SPM system was built based on an off-axis digital holography setup, which enables fast acquisition of phase images. For illumination, we used a collimated laser beam from a He-Ne laser (l = 633 nm). The angle of illumination at the sample plane was varied using a double-axis galvanometer mirror (Cambridge Technology) that was installed at the conjugate plane to the sample plane. The high-N.A. condenser lens (1.4 N.A.; Nikon) and objective lens (1.4 N.A., UPLSAPO; Olympus) enabled the angular coverage of up to 60° with respect to the optical axis. The spiral path of the focused beam enabled fast scanning of the entire N.A., and a complementary metal-oxide semiconductor (CMOS) camera (1024PC; Photon) captured 400 images within less than a second.

Data Analysis. A custom-built MATLAB (MathWorks) code was used to retrieve the scattered light fields, both amplitude and phase delay, from the raw interferogram images acquired with the CMOS camera (27). The phase images at varying incident angles were mapped in the 2D spatial frequency
plane using Eq. 1. The inverse Fourier transform of the 2D mapping (Eq. 2) provides the areal density of a sample, the integral of which provides cell dry mass. The cell boundary was detected with thresholding and manual selection. The probability density and cumulative density functions of dry mass for each group were obtained using a custom-built code for kernel density estimation.

**Cell Culture and Sample Preparation.** L1210 mouse lymphoblasts were grown in Leibovitz’s L-15 CO2 independent media (21083-027; Invitrogen) supplemented with 10% FBS (10438026; Invitrogen), 1 g/L L-(−)-glucose solution (G8270; Sigma-Aldrich), and 1% (vol/vol) 100x penicillin/streptomycin solution (15140-122; Invitrogen). Before each measurement set, L1210 cells from an exponentially growing population were cultured on a 40-mm coverslip precoated with poly-L-lysine (P8920; Sigma-Aldrich). HT-29 and RKO colorectal cancer cells were cultured in DMEM (21063-029; Invitrogen) supplemented with 10% FBS and 1% 100x penicillin/streptomycin solution. At 70–80% confluency, cells were passaged and seeded on a coverslip and then incubated with 1:1 PBS and Polystryrene beads (1 μm) were purchased from Polysciences, Inc. (64030-15).

**On-Stage Cell Culture System.** Cells were observed while grown in a temperature-controlled flow chamber (RC-31; Warner Instruments). The oxygen concentration and pH within the chamber were maintained by the continuous flow of conditioned media preequilibrated with air containing 5% CO2.

The flow rate of the media was determined to guarantee that the rate of oxygen supply was twice as large as the rate of oxygen consumption by the cells inside the chamber. The flow speed inside the chamber was independently controlled by changing the thickness of a spacer, which defines the volume of the chamber. The flow speed was small enough to not disturb cell growth or division (shear stress at wall <1 mPa). A heater embedded in the chamber maintained the temperature of the entire system at 37 °C. Line heaters (MCL-HLS-1; Bioscience Tools) were wrapped around the condenser and objective lenses to minimize heat loss through the immersion oil that is in direct contact with the lenses.

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