Periodicity of nuclear morphology in human fibroblasts

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Motivation: Morphology of the cell nucleus has been used as a key indicator of disease state and prognosis, but typically without quantitative rigor. It is also not well understood how nuclear morphology varies with time across different genetic backgrounds in healthy cells. To help answer these questions we measured the size and shape of nuclei in cell-cycle-synchronized primary human fibroblasts from 6 different individuals at 32 time points over a 75 hour period.

Results: The nucleus was modeled as an ellipsoid and its dynamics analyzed. Shape and volume changed significantly over this time. Two prominent frequencies were found in the 6 individuals: a 17 hour period consistent with the cell cycle and a 26 hour period. Our findings suggest that the shape of the nucleus changes over time and thus any time-invariant shape property may provide a misleading characterization of cellular populations at different phases of the cell cycle. The proposed methodology provides a general method to analyze morphological change using multiple time points even for non-live-cell experiments.

Introduction

Whether form follows function or function follows form is an ongoing debate in biology. Nuclear shape is known to play a role in mechanotransduction, in which cells convert physical forces into chemical signals through connections between the cytoskeleton, nuclear envelope and lamina.⁴ Recent results by Rangamani et al. suggest that changes in cell shape induce local gradients of receptors or signaling molecules which amplify signals during differentiation.¹⁵ Nuclear shape may play a similar signaling role in transcription and other cellular processes. To confirm and understand such phenomena, the first step is studying how nuclear morphology changes over time.

The shape of the nucleus is tightly regulated and changes as a cell differentiates, generally starting out spherical and ending more oblong. Abnormal shape and size are linked with a number of diseases such as cancer and progeria.⁸ Misshapen or lobulated nuclei are used to identify cancerous tissue and estimate cancer grade.⁹ Lamin gene mutations, called laminopathies, lead to misshapen nuclei and cause muscular dystrophy or premature aging in the case of progeria by disrupting the structural network they form around the nuclear periphery.⁴

Several studies have compared nuclear shape under different conditions, such as diseased versus healthy or differentiated vs. undifferentiated.⁶,⁷ None have examined how nuclear morphology normally varies over time within a cellular state (e.g. healthy fibroblasts). Our paper considers primary human fibroblasts synchronized to start at G1 in the cell cycle. We created a program which fits ellipsoids to data from confocal image stacks, then analyzed the resulting shape properties to test the statistical significance of their time variation and extract periodic behavior.

The cell cycle is an obvious explanation for changes in nuclear shape: as the cell grows leading to replication and then cell division, the volume of the nucleus might be expected to increase and then decrease. It is also known that the cell rounds during mitosis as the spindle poles form, allowing chromosomes to line up for division.⁹ For primary human fibroblasts, the cell cycle lasts between 16 and 28 hours with a mean of 20 hours.¹⁹ Recent exploration of transcription factories and the hypothesis that genes physically move into and out of these regions as their expression levels change suggests that cyclically-expressed genes might be an additional cause for changes in nuclear shape.¹⁶ One such set are clock genes controlling circadian rhythm for which a 24 hour or longer cycle would be expected.¹⁸ Another set are genes related to ultradian rhythms for which we would expect to shorter periods often of 8-10 hours.¹⁷

In this study, we probed the unperturbed shape of the nucleus over 75 hours in cell-cycle synchronized primary human fibroblasts from six different individuals. Fibroblasts were chosen because of their applications to cellular reprogramming, wound healing, and ease of access.⁵,¹²,²⁰ Nuclei were stained with DAPI at 32 time points and then captured with 3D confocal microscopy. By modeling the nucleus as an ellipsoid, we derived simple time-varying shape properties
including volume and eccentricity (i.e., roundness or flatness). This is an extension from most current methods that only calculate volume without other shape parameters. The resulting data was then fit to a range of frequencies one of which was consistent with the cell cycle. We found two peak frequencies to extract its primary oscillations. We then fit to a range of frequencies one of which was consistent with the cell cycle. We found two peak frequencies to extract its primary oscillations. We then fit to a range of frequencies one of which was consistent with the cell cycle. We found two peak frequencies to extract its primary oscillations.

Figure 1. Image segmentation and ellipsoid fitting. (A) A single xy slice from a raw z-stack containing multiple DAPI-stained nuclei. (B) An xy slice with xz and yz projections after segmentation into an individual nucleus. (C) Ellipsoidal fit described by the lengths of three axes. Fits of 20 nuclei for each individual and time point were then analyzed.

Algorithms

Ellipsoidal modeling

We built a general analysis tool which fits ellipsoids to 3D volumetric data. Although the tool can handle multiple ellipsoids, in this case each volumetric dataset is cropped to contain just a single nucleus, to which a single ellipsoid is fit. As well as allowing visualization in 3D of a nucleus and its fitted ellipse, shown in Figure 1, the tool’s outputs are the lengths and directions of the ellipsoid’s three primary axis, from which other properties such as volume and eccentricity are easily calculated. To reduce background noise, we clamped all pixels with an intensity less than 1% of the maximum to 0. The set of images from each nucleus was considered as a 3-dimensional volumetric distribution, denoted \( P(p) \), with a fluorescent intensity associated with each 3D position \( p \). Point \( p_i = (x_i, y_i, z_i) \) represents a sample (i.e., pixel in a single z-slice image, dot in right side of Figure 1B) in the volumetric grid with corresponding intensity \( w_i \).

Our method is based on the data’s first- and second-order moments (mean and covariance). It is similar to alternatives such as Gaussian mixture models, but models intensity using a quadratic rather than exponential function. In brief, our method first computes the quadratic distance function best representing the falloff in measured intensities over the nuclear volume, and then optimally thresholds this distance to yield an approximating 3D ellipsoid.

Squared distance at an arbitrary point \( p \) is defined as

\[
D^2(p, Q) = (p - \bar{o})^T Q^{-1} (p - \bar{o})
\]  

where \( \bar{o} \) is the distance origin and \( Q \) is a symmetric, positive definite \( 3 \times 3 \) matrix. Expressing \( Q \) in an eigen-decomposition yields

\[
Q = R_Q \begin{bmatrix}
a^2_Q & 0 & 0 \\
0 & b^2_Q & 0 \\
0 & 0 & c^2_Q
\end{bmatrix} R_Q^T
\]  

where \( a^2_Q, b^2_Q, \) and \( c^2_Q \) are the eigenvalues (representing axis scale factors), and \( R_Q \) is a \( 3 \times 3 \) rotation matrix, representing axis directions. \( Q \) (in boldface) denotes the set of all parameters determining the anisotropic distance metric:

\[
Q = (o, Q) = (\bar{o}, R_Q, a_Q, b_Q, c_Q).
\]

Unconstrained minimization of Eq. 2 causes \( a_Q, b_Q, \) and \( c_Q \) to increase without bound. We therefore constrain the anisotropy so that the sum of axis scale factors raised to some power equals the dimensionality:

\[
a^\gamma_Q + b^\gamma_Q + c^\gamma_Q = 3.
\]  

Note that the identity transformation \( (Q = I) \) satisfies this constraint. As \( \gamma \to 0 \), anisotropy is unconstrained; highly eccentric shapes like needles or pancakes are freely permitted. As \( \gamma \to \infty \), the ellipsoid is forced to be completely spherical. We used the normalization power \( \gamma = 1 \) to balance between these extremes for robust model fitting.

We then seek the \( Q \) yielding minimal sum of intensity-weighted squared distances over \( P \)

\[
Q = \arg \min_Q \left( \sum_{p_i \in P} w_i D^2(p_i, Q) \right)
\]  

subject to the constraint in Eq. 3. It can be calculated in terms of the volumetric data set’s mean vector, \( \bar{p}(P) \), a weighted average of the dataset, and covariance matrix

\[
C(P) = \frac{\sum_{p_i \in P} w_i (p_i - \bar{p}) \otimes (p_i - \bar{p})}{\sum_{p_i \in P} w_i},
\]

See the supplement for the detailed derivation of optimal ellipsoid, which we summarize in the following.

The optimal origin in \( Q \) is given by the mean of the data set: \( o = \bar{p}(P) \). Let the covariance matrix, \( C(P) \), be decomposed into
its eigenvectors and eigenvalues via

$$C(P) = R_C \begin{bmatrix} a_C^2 & 0 & 0 \\ 0 & b_C^2 & 0 \\ 0 & 0 & c_C^2 \end{bmatrix} R_C^T$$  \[6\]

The method of Lagrange multipliers can then be used to show that the optimal $Q_1$ has rotation identical to the covariance’s eigen-rotation; that is, $R = R_C$. The optimal scale factors $a_C^2$, $b_C^2$, $c_C^2$ are proportional to the exponentiated eigenvalues of $C(P)$ via

$$a_C^2 = \alpha a_C^{4/(\gamma + 2)},$$
$$b_C^2 = \alpha b_C^{4/(\gamma + 2)},$$
$$c_C^2 = \alpha c_C^{4/(\gamma + 2)},$$  \[7\]

where

$$\alpha = \frac{3}{\left(\frac{2\gamma}{a_C^{2\gamma/(\gamma + 2)} + b_C^{2\gamma/(\gamma + 2)} + c_C^{2\gamma/(\gamma + 2)}}\right)^{2/\gamma}}$$  \[8\]

and, as mentioned earlier, we fix $\gamma = 1$. An approximating ellipsoid can then be computed from this anisotropic distance metric by thresholding squared distance via $D^2(p, Q_1) \leq d^2$, for some appropriate threshold $d$. It is computed so that an arbitrarily-scaled version of the binary function

$$B(p, Q_1, d) = \begin{cases} 1 & \text{if } D^2(p, Q_1) \leq d^2, \\ 0 & \text{otherwise,} \end{cases}$$  \[9\]

has least squared error compared to the actual volume of intensities $w_i$. More precisely,

$$d_s = \arg \min_d \sum_{p_i \in P} (\tau B(p_i, Q_1, d) - w_i)^2,$$  \[10\]

where the optimal scale factor $\tau$ is given by

$$\tau = \frac{\sum_{p_i \in P} B(p_i, Q_1, d) w_i}{\sum_{p_i \in P} B(p_i, Q_1, d)}.$$  \[11\]

Note that we can remove the square in the denominator above usually present in least-squares projection because $B$ is a binary function, so $B^2 = B$. Finally, the lengths of the approximating ellipsoid’s three axes are given by

$$a = d_s a_s^{-1},$$
$$b = d_s b_s^{-1},$$
$$c = d_s c_s^{-1}.$$  \[12\]

Period estimation

We examined six measures of nuclear shape including volume from ellipsoid fitting, $V = \frac{4\pi}{3} a b c$, volume by direct counting of voxels whose intensity exceeds a threshold, and eccentricity, $e = \sqrt{1 - c^2/a^2}$, where $c$ is the shortest and $a$ the longest ellipsoid axis. Eccentricity reflects the roundness or flatness of a shape; a sphere has an eccentricity of 0 while a needle or pancake shape has an eccentricity close to 1. Finally, we measured the three axis lengths themselves, yielding a total of 6 different shape properties. Sampling each property over time for one of the six individuals yields a time series $f_i$, $i = 1, 2, \ldots, n$, sampled at $n = 32$ different time points denoted $t_i$. These times were not sampled uniformly over the 75 hours (see Table 1), complicating spectral analysis. The non-uniform sampling and limited number of time points made Fourier analysis ineffective. Each time series value, $f_i$, was calculated by averaging over the 20 cell nuclei sampled per time point.

To extract prominent frequencies in these time series, we fit them to a single-frequency pair of harmonic basis functions using least squares, considering frequency as a continuous parameter. We first centered the data by subtracting the mean of each time series, giving $\tilde{f}_i$, $i = 1, 2, \ldots, n$. Given a frequency $\omega$, we minimized the mean squared error (MSE) between the resulting data, $\tilde{f}_i$, and the basis functions.

Table 1. Sampling Schedule. The time points imaged and analyzed for each of the six individuals.

| time index $i$ | time from start $t_i$ (hr) | interval $t_{i-1} - t_i$ (hr) |
|----------------|-----------------------------|-------------------------------|
| 1              | 5                           | 5                             |
| 2              | 8                           | 3                             |
| 3              | 11                          | 3                             |
| 4              | 14                          | 3                             |
| 5              | 17                          | 3                             |
| 6              | 20                          | 3                             |
| 7              | 23                          | 3                             |
| 8              | 26                          | 3                             |
| 9              | 29                          | 3                             |
| 10             | 31                          | 2                             |
| 11             | 33                          | 2                             |
| 12             | 35                          | 2                             |
| 13             | 37                          | 2                             |
| 14             | 39                          | 2                             |
| 15             | 41                          | 2                             |
| 16             | 43                          | 2                             |
| 17             | 45                          | 2                             |
| 18             | 47                          | 2                             |
| 19             | 49                          | 2                             |
| 20             | 51                          | 2                             |
| 21             | 53                          | 2                             |
| 22             | 55                          | 2                             |
| 23             | 57                          | 2                             |
| 24             | 59                          | 2                             |
| 25             | 61                          | 2                             |
| 26             | 63                          | 2                             |
| 27             | 65                          | 2                             |
| 28             | 67                          | 2                             |
| 29             | 69                          | 2                             |
| 30             | 71                          | 2                             |
| 31             | 73                          | 2                             |
| 32             | 75                          | 2                             |

Figure 1 shows an example.
\[ \alpha \sin \omega t_i + \beta \cos \omega t_i, \]
by solving a 2 x 2 linear system in the coefficients \( \alpha \) and \( \beta \):

\[
\alpha \sum_{i=1}^{n} \sin^2 \omega t_i + \beta \sum_{i=1}^{n} \sin \omega t_i \cos \omega t_i = \sum_{i=1}^{n} f_i \sin \omega t_i,
\]

\[
\alpha \sum_{i=1}^{n} \sin \omega t_i \cos \omega t_i + \beta \sum_{i=1}^{n} \cos^2 \omega t_i = \sum_{i=1}^{n} f_i \cos \omega t_i.
\]

In addition to looking at prominent frequencies, the phase of the basis function, \( \theta = \tan^{-1}(\alpha/\beta) \), reflects the relative timing.

The spectrum was represented by the amount of squared energy accounted for by this fit; that is, the difference between the squared signal energy in the original time series data and the squared residual (unfit) energy, given by

\[
F(\omega) = \sum_{i=1}^{n} f_i^2 - (f_i - \alpha \sin \omega t_i - \beta \cos \omega t_i)^2.
\]

We then swept the basis function frequency \( \omega \) and looked for peaks in \( F(\omega) \), indicating a relatively good fit at that frequency. To reduce noise and identify frequencies prevalent across individuals, we also plotted the average fit power, \( F \), over all six individuals.

Our method is similar to the Lomb periodogram, a standard procedure for analyzing periodicities in an irregularly-sampled time series. It is a simple extension of the related least-squares spectral analysis which unlike Lomb analysis keeps the phase information of the basis functions. Our method differs by directly evaluating the squared signal energy represented by the basis rather than its squared coefficients, \( \alpha^2(\omega) + \beta^2(\omega) \).

**Methods**

**Sample preparation**

Human primary fibroblasts from six normal male newborns (discarded foreskin tissue, passage 3) were cultured in complete media: MEM medium (Life Technologies, 10370-088) supplemented with 10% fetal bovine serum (VWR, SH30071.03), 2 mM L-glutamine (Life Technologies, 25300081), and 1 x Antibiotic-Antimycotic (Life Technologies, 15240-062), at 37 °C with 5% CO₂. On the day before the experiment, cells were trypsinized, 5 x 105 of them re-suspended in 15 ml complete media as described above, and seeded into T75 flasks. Inspecting cells under the microscope 24 hours later, confluency with its likely inhibition of cell division.

Cells were washed with 15 ml pre-warmed PBS twice, and serum-free MEM medium (2 mM L-glutamine, and 1X Antibiotic-Antimycotic) was added to each flask to begin cell synchronization. Cells were incubated at 37 °C with 5% CO₂. After 24 hrs, each flask of cells were re-suspended with 2 mls 0.25% trypsin-EDTA, followed by 10 mls of complete media to inactivate the trypsin treatment, centrifuged at 750 rpm for 5 minutes, and each cell set re-suspended in 12 mls of serum-free media. Cell counts were performed, and 150 mls (~ 30K cells) placed on Fisher superfrost slides in petri dishes. Cells were allowed to settle for 2 hrs at 37 °C with 5% CO₂. 15 mls of complete media were then added to each Petri dish, sample slides taken at the time shown in Table 1, rinsed briefly in PBS, fixed in 4% paraformaldehyde for 8 minutes, and rinsed 3 x 5 minutes in PBS. 15 μl of Prolong Gold (p36941 Life Technologies) with DAPI was placed on each slide, an 18 x 18 mm coverslip applied, sealed, and stored at -20 °C until imaging. The sample from individual 3 at time point 5 was unusable due to a lack of cells. All imaging was completed on a Zeiss LSM 710 Microscope with a 63 x Oil DIC objective, 0.2 μm x and y resolution, 0.5 μm z resolution, an oversampled pixel size of 0.132 μm x and y and 0.320 μm z, and 24 μm pinhole. Excitation was by a 405 nm laser with an emission collection band from 411 to 486 nms.

A volumetric dataset for each individual nucleus was formed by cropping a z-stack of images from confocal microscopic measurements. Ellipsoidal approximation (described in the Algorithms section) was performed on 20 nuclei from each individual at each time point.

**Volume verification by thresholding**

We used MATLAB to calculate the volume of nuclei by thresholding to validate our ellipsoid fitting. The 3D images were loaded into MATLAB, and thresholded with a cutoff of 5% of the maximum. Any holes that could not be reached from the outside of the image were filled to prevent nucleoli or other internal structures from being missed. The volume of all selected pixels was then integrated over all images in the z-stack. Images from 20 nuclei for each individual and time point were analyzed as was done for the ellipsoid fitting. To verify that the choice of threshold did not determine the periodic results, we tried multiple thresholds (0.04, 0.045, 0.05, 0.055, 0.06, 0.065) and calculated spectrums for each.

**Bootstrapping**

To see how consistent our periodicity results were, we took 100 random samples with replacement (bootstrapping) of half the data (10 out of 20 nuclei from each time point and individual) and used this limited data set to rerun the periodicity calculations described in “Period Estimation.”

**Variance analysis over time**

We used one-way analysis of variance (ANOVA) to show that changes over time in volume or eccentricity cannot adequately be explained by sampling from a single distribution. Each time point was considered a category with 20 observations (nuclei) and the test was run separately on each of the six individuals. A p
-value of .05 was used to test the null hypothesis that all of the variability seen between time points was due to random chance and that the eccentricity and volume were each drawn from a single distribution. The alternate hypothesis was that the distributions changed over time. Bonferroni correction was used to account for multiple tests: 6 individuals × 2 properties.¹

## Results and Discussion

**Ellipsoid model captures variability in nuclear shape**

We analyzed nuclear shape and volume of primary human fibroblasts that had been cell-cycle synchronized by 2-days serum starvation. Nuclear shape and volume were analyzed at 32 time points over a 75 hour period (time points shown in Table 1) for cells from six different individuals. For each time point and individual, confocal microscopy was used to get 3D volumetric distributions of 20 nuclei stained by DAPI. An analysis and visualization tool performed the ellipsoidal approximation for each nucleus and calculated the three lengths of the ellipsoid axes as shown in Figure 1.⁶

The lengths of the three axes, a, b, and c, were then used to calculate each nuclei’s volume, \( V = \frac{4}{3}abc \), and eccentricity, \( e = \sqrt{1 - c^2/a^2} \).

We also calculated volume independently by counting non-zero voxels after thresholding the images in MATLAB, yielding a total of six different shape indicators. In Figure 2A, xy and xz projections of the time course for each individual and the average of the individuals show fluctuation in the nuclear shape over time. The ellipsoid volume, threshold volume and ellipsoid eccentricity are also shown over the time course in Figure 2B, 2C, and 2D respectively. The average for each feature and individual is listed in Table 2.

³⁶This tool was originally developed to spatially approximate homologous chromosome territories in the nucleus, where a pair of such territories was expected to be present simultaneously in the volumetric data. We applied the same tool to approximate an entire nucleus as a single ellipsoid.

| Table 2. Average ± standard deviation for each feature and individual. |
|--------------------------|--------------------------|--------------------------|
| **Threshold** | **Ellipsoid** | **Eccentricity** |
| **Volume (\( \mu m^3 \))** | **Volume (\( \mu m^3 \))** |                         |
| Sample 1 | 951 ± 387 | 649 ± 352 | 0.949 ± 2.47<e> – 4 |
| Sample 2 | 877 ± 299 | 492 ± 301 | 0.967 ± 9.20<e> – 5 |
| Sample 3 | 960 ± 464 | 883 ± 519 | 0.941 ± 8.37<e> – 5 |
| Sample 4 | 872 ± 332 | 748 ± 305 | 0.943 ± 6.91<e> – 5 |
| Sample 5 | 1090 ± 488 | 836 ± 406 | 0.940 ± 1.19<e> – 4 |
| Sample 6 | 1300 ± 608 | 1010 ± 497 | 0.936 ± 1.17<e> – 4 |
| All Samples | 1010 ± 466 | 769 ± 438 | 0.946 ± 1.49<e> – 4 |

| **Axis 1 (\( \mu m \))** | **Axis 2 (\( \mu m \))** | **Axis 3 (\( \mu m \))** |
|--------------------------|--------------------------|--------------------------|
| Sample 1 | 2.55 ± 0.691 | 6.68 ± 1.32 | 8.56 ± 1.20 |
| Sample 2 | 2.02 ± 0.620 | 6.42 ± 1.37 | 8.36 ± 1.32 |
| Sample 3 | 2.91 ± 0.397 | 7.61 ± 1.56 | 8.87 ± 1.70 |
| Sample 4 | 2.78 ± 0.376 | 7.22 ± 1.09 | 8.55 ± 1.20 |
| Sample 5 | 2.91 ± 0.400 | 7.40 ± 1.34 | 8.76 ± 1.48 |
| Sample 6 | 3.17 ± 0.606 | 7.83 ± 1.34 | 9.15 ± 1.40 |
| All Samples | 2.72 ± 0.644 | 7.19 ± 1.43 | 8.71 ± 1.41 |
Nuclear shape changes over time

To show that the fluctuations in nuclear size and shape, seen in Figure 2, cannot be explained by random chance, we performed one-way analysis of variance (ANOVA) on the data for three shape properties: ellipsoidal volume, thresholded volume, and eccentricity. ANOVA tests whether data from different categories, in this case time points, can be explained by a single distribution or whether it requires different per-category distributions. Box plots of the distributions for each individual are shown in Figures S1, S2, and S3 respectively. The null hypothesis was that a single distribution explains all of the variability. We were able to reject the null hypothesis at a $p \leq 0.05$ level for all three shape properties (eccentricity, ellipsoidal volume, and thresholded volume) and for all individuals.

Periodicity of the nuclear shape matches cell cycle and circadian rhythm timing

By fitting a set of single-frequency basis functions to mean-centered ellipsoid volume, thresholded volume, eccentricity, and three axis lengths, as described in “Period Estimation,” we computed the extent to which each was fit by a range of different frequencies. Figure 3B shows how well each parameter was fit by the basis functions for each individual. Results varied significantly over individuals, but a few frequencies were seen consistently across the six individuals and six nuclear shape indicators. The most prominent peak, marked with red dots on Figure 3A, has a mean of 17.3 hours (min 16.5, max 18.3) and is the highest peak in the spectrums for all of the six measures. For the two measures of shortest axis length and eccentricity, it was the second highest peak.

Best fit basis functions for each shape property are shown in Figures 4 A-F. Table S1 reports the basis function parameters we calculated, along with normalized MSE (NMSE, meaning MSE divided by the mean squared energy of the original signal), and peak signal-to-noise ratio (PSNR). The fit for eccentricity, shown in Figure 4B, has a 17 hour period and matches the data fairly well, yielding NMSE of 0.0045 and PSNR of 12.7. Such a low value for NMSE and high value for PSNR indicates that the fit captures much of the variability in the time series, and supports the hypothesis that this prominent peak reflects the cell cycle. Cells become rounder during mitosis so we expect a dip in eccentricity as mitosis begins and an increase after the cells finish dividing. In addition, nuclear volume is expected to increase through the cell cycle leading up to division. We looked at the phase of the top fits (all those included in Tables S1 and S4) that had periods according with the cell cycle (15-22 hours) and found that the eccentricity had an average phase of 0.6645 rad,
meaning it peaked 1.5 hours after serum was returned to the cells
(and every \( \pm 17 \) hours after that). The average phase of the volume
was \(-0.1143\) rad, meaning it peaked about 10 hours after serum
was re-added to the cells. Both observations are consistent
with an initial stalling of the cells in the G0/G1 phase due to a
lack of serum, followed by later attainment of maximal volume
as the cells prepare to divide.

Across individuals and shape features, we also observed a second period at roughly 26 hours
(min 23.5, max 28.9). This peak was weaker than the 17.3 hour cycle in all shape measures except
for the shortest axis length. It could be a result of the circadian rhythm that controls humans’
internal clock and sleep schedule. Although not as prevalent as the cell cycle period across all individuals and shape features, it yielded fits with a median
NMSE of 0.0041 and PSNR of 15.5 for the individuals and shape features for which it was one of the
top two peaks, indicating an even better fit there than the first (cell cycle) period.

The spectrum for thresholded volume, based on a threshold of
5% of the data set’s maximum intensity, is mostly consistent
with the other ellipsoid-based shape parameters and includes
peaks at both of the above frequencies. However, the spectrum
also includes a peak (in fact its tallest) not seen in the other spectrums at 11.3 hours. Oscilla-
tion at a single frequency explains the observed time variation only imperfectly, suggesting
that multiple complex traits affect nuclear shape. In addition,
although the cells are initially synchronized to the same place
in the cell cycle, natural variation in cell division time leads to pro-
gressively less synchronization over the 75 hours. Different
individuals are not necessarily in the same phase; see Tables S1
and S4. They are almost all within the same half cycle, but
each peak at different times. This may be due to a combination of
1) differences across individuals
in the time needed for cells in serum to return to growth
and therefore begin dividing, and 2) progressively degrading
synchronization over the roughly four cell cycles within our
observation window.

We also tried other volume thresholds (0.04, 0.045, 0.055, 0.06, 0.065) and found they agreed better with peri-
oids extracted from other shape properties. We observed peaks

Figure 4. Optimal fits over all individuals. The blue lines are the average of the mean-centered data for A) volume, B) threshold volume C) eccentricity, D) longest, E) middle, and F) shortest axis lengths. The red lines show the fit at the strongest detected frequencies of 18.3, 17.1, 16.5, 17.1, and 24.6 hours, respectively. G-L show histograms recording the top two peaks from each of 50 random samples of half the data for G) volume, H) threshold volume, I) eccentricity, J) longest, K) middle, and L) shortest axis lengths, respectively.* **
at the same three frequencies in all cases (see Figures S6 and S7). In fact, the 0.05 threshold was the only one in which the 11.3 hour peak was tallest. In four of the alternate thresholds, the 11 hour peak was second tallest after the 17 hour peak, and in one it fell below both the 17 and 26 hour peaks.

To verify that these periods were not accidental, we used bootstrapping (random sampling with replacement of 10 out of 20 nuclei per time point) and extracted dominant periodicities from this data subset. After doing this 100 times, we made histograms of the top 2 peaks seen in each sample for each of the six shape features. Five examples of the dynamics and spectrums of these random samples are shown in Figures S4 and S5. As seen in Figures 4 G-L, the histograms all have strong peaks in the 14-18 hour bin as well as another split between the 22-26 and 26-30 hour bins depending on the feature. These peaks are weakest in panel H, corresponding to thresholded volume, as it is dominated by a peak in the 10-14 hour bin, consistent with the peak at 11.3 hours observed across the full dataset.

**Conclusion**

Using a simple model of nuclear shape in which the nucleus is modeled as an ellipsoid represented by its three axis lengths and derived from DAPI-stained images, we find that both the eccentricity and volume of primary human fibroblast nuclei change significantly over time. A single sample in time provides an incomplete picture. This result has significant impact for studies comparing cell populations, where normal time variation can be conflated with differences between cell types. Observations at multiple time points seem to be necessary to establish that any size or shape differences are due to intrinsic differences rather than natural oscillations. By comparing multiple nuclei sampled at different time points, conclusions can be drawn about the dynamics of nuclear shape without measuring it as a continuous property in a single cell.

The methodologies provided in this paper are straightforward and simple to apply. Our statistical methodology has the benefit of being applicable to non-live (i.e. sacrificial) protocols. Although the initial steps in data analysis required some user input, they can easily be fully automated to simplify future studies or translational work.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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