Validation of spatiotemporal image correlation spectroscopy (STICS) for the measurement of diffusion

By

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ABSTRACT

Understanding of chemical mechanisms on an intracellular level is steadily growing and extensively studied. However, our understanding of the physical bases for these processes is comparatively lacking. Current limitations on measuring fundamental intranuclear mechanical properties have stifled necessary new perspectives on intranuclear mechanics. Here we build on the Wiseman group’s novel development of spatiotemporal image correlation spectroscopy (STICS) to measure diffusion, and then attempt to validate it with simulated and experimental datasets. STICS, unlike current methods of diffusion measurement, such as image mean square displacement (iMSD), does not require the ability to track distinct particles. Expected diffusion coefficients, $D$, in simulated datasets were calculated using the Stokes-Einstein relation, which relates the diffusive properties of particles moving in fluids as $D = \frac{k_B T}{6 \pi \eta r}$. For experimental bead diffusion datasets expected $D$ values were measured using iMSD. We observe that in simulated datasets STICS is able to measure a linear relationship between $1/D$ and the viscosity, $\eta$, as expected by Stokes-Einstein. We also see that STICS has a high precision, as $D$ measured from subsamples of a given video have an average standard deviation of $3.23 \times 10^{-34} \text{m}^2/\text{s}$. However, in both simulated and experimental datasets the measured $D$ value, denoted $D_S$, differs from the expected $D$ by a calibration factor $D = \beta D_S$ that appears to depend on particle density, but could also depend on signal intensity, bleaching rates, and/or imaging plane thickness. Future work will be aimed at characterizing $\beta$ and thus essentially calibrating STICS, since at present this variability makes it impractical to measure diffusion using STICS.
I. INTRODUCTION

While our understanding of chemical mechanisms on an intracellular level is steadily growing and extensively studied, our understanding of physical processes is comparatively lacking. For example, multiple mechanisms of chemical signal transduction into the nucleus have been proposed and identified, such as G coupled proteins and other second messenger cascades (Lodish et al., 2000). However, mechanotransduction, the transfer of physical stimuli into the cell, is a much less understood topic (Alenghat and Ingber, 2002). Furthermore, genomic sequencing has facilitated our understanding of gene regulation by nucleotide sequences, but we are only beginning to scratch the surface of other regulatory elements, such as structure, physical arrangement, and perhaps most interestingly, force (Li and Reinberg, 2011). We have reason to believe that physical mechanisms such as those mentioned above do exist and serve functional roles - physical forces have repeatedly been correlated with cell behavior, ranging from stem cell differentiation to metastasis and gene expression (Wirtz et al., 2011; Dado et al., 2012). In addition, the literature provides evidence of structural components such as actin cytoskeletons ‘transmitting signals’ and affecting cell behavior in response to physical stimuli (Schwarz and Gardel, 2012). In order to further elucidate the workings of physical mechanisms, it is necessary to understand physical properties on a fundamental level, such as stress, strain, elasticity, tensile and compressive forces, flow, and diffusion. Of strong interest to us is the measurement of diffusion, which can be used to decipher numerous higher-level physical phenomenon, such as free and active transport, structural heterogeneity within the nucleus, and the release of bound proteins upon stimulation.
The experimental setup we are building towards for the measurement of these fundamental physical properties utilizes atomic force microscopy (AFM) coupled with vertical light sheet microscopy (VLSM) to image cells under induced forces. AFM is used for the repeated induction of compressive and adhesive forces, used similarly in prior studies to measure physical properties of live cells (Gavara and Chadwick, 2012; Raman et al., 2011). The technique of VLSM accomplished through the use of PRISM imaging allows imaging in the plane of deformation on a time scale that can capture relevant physical information, an advantage over the use of confocal microscopy to build 3D stacks. This approach facilitates the capture of images containing data on diffusion, flow, strain, and stress, and to extract these data we chose to use spatiotemporal image correlation spectroscopy (STICS). The STICS code we built on was made by the Wiseman group as the one of the first steps in developing STICS as a novel analysis tool to measure flow (Herbert and Wiseman, 2005).

Current methods of measuring diffusion and flow are based on tracking individual particles, as in iMSD and particle tracking velocimetry (PTV), or in correlation techniques such as particle image velocimetry (PIV) (Ribeiro et al., 2015; Holenberg et al., 2012; Amira et al., 2013). However, all of these techniques require the capability to image distinct individual particles, a condition that makes it difficult to use small-molecule labels, such as histone markers, in high particle density situations. Furthermore, PIV cannot be easily used to measure diffusion – its functionality is limited primarily to measuring flow. This needed function is served by STICS, which correlates regions of the image with applied temporal and spatial shifts, generating meshes of velocity vectors and diffusion coefficients across each frame of a video. (Hebert et al., 2005). Meshes of
data on such a small scale allow us to study the heterogeneous nature of the nucleus, potentially identifying regions of abnormal flow, diffusion, and/or strain.

Given the novelty of STICS, it is important to validate the analysis using known simulated and experimental datasets. It has only recently been developed by the Wiseman group, and has only been used in a handful of papers (Hedde et al., 2014). Prior work within our lab had been done to validate the use of STICS for the measurement of flow in situations resembling an AFM coupled VLSM setup, but the measurement of diffusion remained to be implemented and validated.

The goal of this project was thus to implement and validate the use of STICS as a tool to measure diffusion for future experiments in which forces are induced onto cell nuclei. Specifically, I built upon the implementation of STICS from the Wiseman lab to include the capability to measure the diffusion of subpixel particles in situations resembling AFM coupled VLSM experiments. These situations include particle densities greater than those analyzable by current methods, and range in viscosities from 1 to 300 cP, the observed range of viscosity in the cell cytoplasm and vesicles (Kuimova et al., 2009). I validated the measurements on simulated datasets generated from existing code, as well as experimental datasets I designed and analyzed using iMSD code I built and verified. Resulting measurements show that STICS is able to measure a linear increase in the inverse diffusion coefficient, $1/D$, with an increase in viscosity, as well as measure $D$ with high precision, but its accuracy is off by a calibration factor, $\beta$, whose origin is yet to be determined.
II. METHODOLOGY

Implementing STICS

An implementation of STICS from the Wiseman lab was built upon to include the capacity to measure diffusion. STICS utilizes a generalized intensity fluctuation correlation function (equation 1) to compute a correlation score, $r$, as a function of spatial and temporal lags for a given region of interest defined by $x$, $y$, and $t$. Specifically, $\zeta$ and $\eta$ represent spatial lags and $\tau$ represents a temporal lag (Hebert and Wiseman, 2005).

$$ r(\zeta, \eta, \tau) = \frac{\langle \delta i(x, y, t) \delta i(x + \zeta, y + \eta, t + \tau) \rangle}{\langle \delta i \rangle \langle \delta i \rangle_{t+\tau}} \quad (Eq. 1) $$

In equation 1, $\delta i(x, y, t)$ represents the intensity fluctuation at $(x,y,t)$, i.e. the signal above the background, with $\delta i(x, y, t) = i(x, y, t) - \langle i \rangle$, and $\langle ... \rangle$ representing spatial averaging at a given time. To extract flow and diffusion information we utilize a discrete approximation that combines spatial correlation information with temporal correlation information (Hebert and Wiseman, 2005).

$$ r'(\zeta, \eta, \Delta t) = \frac{1}{N - \Delta t} \sum_{i=t}^{N-\Delta t} \frac{\langle \delta i(x, y, t) \delta i(x + \zeta, y + \eta, t + \Delta t) \rangle}{\langle \delta i \rangle \langle \delta i \rangle_{t+\Delta t}} \quad (Eq. 2) $$

Here, $N$ is the total number of images in the series, and $r'$ represents the averaged correlation functions over the $(\zeta, \eta)$ plane for all frames separated by the time lag $\Delta t$. The $r'(\zeta, \eta, \Delta t)$ averaged correlation function for a given $\Delta t$ can be fit to a Gaussian of the form

$$ r'(\zeta, \eta, \Delta t) = g_o(\Delta t) \exp \left( \frac{(\zeta - \rho(\Delta t))^2 + (\eta - \psi(\Delta t))^2}{\omega^2(\Delta t)} \right) + g_{offset}(\Delta t) \quad (Eq. 3) $$

where $g_o(\Delta t)$, $g_{offset}(\Delta t)$, $\rho(\Delta t)$, $\psi(\Delta t)$, and $\omega^2(\Delta t)$ are functions of $\Delta t$ and represent the amplitude, the baseline correlation score as $\zeta, \eta \rightarrow \infty$, the $\zeta$ coordinate of the peak’s
center, the $\eta$ coordinate of the peak’s center, and the peak’s width respectively. At a time lag of 0, we see that the Gaussian is centered at $(\zeta, \eta) = (0,0)$. In the case of flow we expect the peak to move in the direction of flow as $\Delta t$ increases, and can calculate flow velocities $v_\zeta$ and $v_\eta$ by tracking the peak locations with respect to $\Delta t$ as shown in equation 4.

$$\rho(\Delta t) = v_\zeta \cdot \Delta t, \quad \psi(\Delta t) = v_\eta \cdot \Delta t \quad (Eq. \ 4)$$

In the case of diffusion we would also expect the peak to become wider as it evolves to larger time lags with the rate of change in width related to the diffusion coefficient, $D$ (Herbert and Wiseman, 2005). In the correlation of $\omega^2$ and $\Delta t$ (Eq. 5) the $\omega_0^2$ represents the width of the Gaussian for a time lag of 0, i.e. the average width when regions are correlated with themselves.

$$\omega^2(\Delta t) = 4D \cdot \Delta t + \omega_0^2 \quad (Eq. \ 5)$$

To calculate diffusion in a sample video the $D$ values are calculated for each ROI in each frame and then averaged together.

The implementation of STICS code we received from the Wiseman group was built to provide flow data, but was not able to provide diffusion data without code alteration. I specifically built in the capacity to measure diffusion by writing the necessary code to extract the widths of the Gaussians and track their evolution with respect to $\Delta t$.

**Simulated data sets**

Data sets were simulated using a bead diffusion simulator built previously in the lab, which was then modified to effectively simulate small particles at high densities by altering the point-spread function as necessary. Specifically, the width of the point-spread function...
function was previously empirically determined for our lab’s microscope setup with particles ranging in diameter from 100nm to 1000nm. For a 10nm bead, the standard deviation of the PSF was treated as the same as the standard deviation for the PSF of the 100nm bead. Particle tracks were determined using a random walk algorithm, and these tracks were then visualized into an image series using the point-spread function previously mentioned. Frames from a sample simulated data set are shown in figure 1.

**iMSD analysis**

Analysis via image mean square displacement (iMSD) was conducted on samples to cross verify the diffusion values measured by STICS and those predicted utilizing Stokes-Einstein theory. Video Spot Tracker (CISMM at UNC-CH) was used to select individual particles, which were then tracked to give frame-by-frame locations. The resulting series of coordinates were used to generate mean square displacement (MSD) vs. $\tau$ plots, and the slope of the fit in equation (6) was used to calculate the diffusion coefficient, $D$.

$$MSD(\tau) = 4D\tau \quad (\text{Eq. 6})$$

In videos with high particle densities and/or noisy conditions, the tracking of particle movement was conducted by hand using FIJI due to the inability of the Video Spot Tracker program to accurately follow beads.

**Computational utilization**

Due to the computational resources required to generate simulated bead datasets and run STICS, jobs were run on the BASS supercomputer at the University of North Carolina at Chapel Hill.
**Experimental bead datasets**

Experimental datasets were created by suspending 20nm, 100nm, and 200nm yellow-green carboxylic acid coated Invitrogen fluorospheres in sucrose solutions. Aliquots of the suspensions were then imaged using a 40x oil objective in a microscope chamber held at 37°C. Frames from a sample experimental bead dataset are shown in figure 2.

**III. RESULTS**

**Findings from simulated datasets**

**Verification of simulation using iMSD**

Image MSD analysis was conducted on simulated datasets to ensure the simulation would provide sample videos with diffusion coefficient values aligning with those predicted by Stokes-Einstein. This agreement is shown in figure 3, in which diffusion values measured by iMSD for various datasets simulated over a range viscosities agree with the predicted Stokes-Einstein values. The deviation from the expected values is likely attributable to the small sample set of beads that could be successfully tracked.

**Ability of STICS to measure linear relationship between 1/D and viscosity**

For initial STICS validation 100nm particles were simulated at varying viscosities, and the resulting videos were analyzed at 100 frames per second (FPS) using STICS. A plot of 1/D versus viscosity in figure 4 shows a linear regime, where an increase in viscosity corresponds to the expected linear increase in 1/D (the regions fitted in figure 4). The linear component is represented as a modified version of Stokes-Einstein

\[
\frac{1}{D} = \left( \frac{\beta \cdot 6\pi r}{k_B T} \right) \eta \quad \text{(Eq. 7)}
\]
where $\eta$ represents viscosity, $r$ represents particle radii, $T$ represents temperature, $k_B$ represents the Boltzmann constant, and $\beta$ represents a calibration factor. For the linear region of the 100 FPS simulations in figure 4 we calculate $\beta = 0.0055$ with a correlation score of 0.95. As shown in figure 4, we note that this linear regimen is extended when the frame rate of the simulation is decreased from 100 FPS to 25 FPS with $\beta$ changing minimally, 5%, to $\beta = 0.0052$ with a correlation score of 0.99.

**Effect of changing particle density**

We then wished to elucidate which parameters influence $\beta$, beginning with particle density. We decided to determine the impact of varying particle density by simulating 10nm particles at 30 particles/um$^2$ and 40 particles/um$^2$ across varying viscosities, the results of which are displayed in figure 5. Again we noticed a linear region, in this case up to $\eta < 0.4 Pa\cdot s$, and fit the data to equation 7. We see that the value of $\beta$ changes with particle density from $\beta = 0.0031 (r = 0.91)$ with 30 particles/um$^2$ to $\beta = 0.0039 (r = 0.96)$ with 40 particles/um$^2$, representing a 25% increase in $\beta$ for a 33% increase in particle density.

**Findings from experimental datasets**

**Differences between theoretical predictions and PIV measurements**

Whereas in the simulated datasets iMSD showed agreement between visualized diffusion and that predicted by Stokes-Einstein, the same does not hold for experimental bead videos. MSD plots for individual 100nm beads in a given video show varying diffusion coefficients (figure 6), and even when averaged across multiple beads to calculate average diffusion coefficients for the video we see differing values between the iMSD calculated values and theoretical predictions (figure 7). These differences in
expected and observed diffusion coefficients could arise from imprecise temperature control, bead clustering affecting effective particle radii, and errors in the viscosity measurements of the standards, all of which contribute to the calculation of $D$ using Stokes-Einstein. Thus, due to the inability to calibrate STICS against values predicted by Stokes-Einstein, we decided to correlate measurements from STICS with those measured by iMSD.

**Linear relationship between diffusion coefficients measured by iMSD and STICS**

Given that we were unable to compare STICS measurements to theoretical values, we compared $D$ values measured by STICS to $D$ values measured by iMSD to determine if measurements in experimental datasets differed from expected values by a calibration factor $\beta$. Bead suspensions were prepared with 20nm, 100nm, and 200nm beads in H$_2$O, 2M sucrose, and 2.5M sucrose solutions at 1% concentrations from the original bead stock solutions. Each suspension was then analyzed using both iMSD and STICS. The resulting values are graphed in figure 8, with the diffusion coefficient as measured by iMSD on the x-axis and the diffusion coefficient measured by STICS on the y-axis. Samples in which either iMSD was not possible due to an inability to effectively track beads or which were too noisy for STICS are not depicted. The resulting plots show linear relationships between $D_S$, the diffusion coefficient value measured by STICS, and $D$, the diffusion coefficient value measured by iMSD, for each set of beads. Specifically, we observe $D = \beta D_S$ where $\beta = 0.075$, $\beta = 3.703$, and $\beta = 0.117$ with correlation coefficients of 0.98, 0.97, and 0.93 for the 200nm, 100nm, and 20nm beads respectively.
Consistency of STICS within subsamples

If the STICS measurements differ from expected values by a calibration factor $\beta$ that depends on the experimental setup, then measurements from subsamples of the same setup should differ by the same $\beta$. To test this, videos of 200nm particles in 2M sucrose at particle densities of 0.1% and 1% (sample frames shown in figure 2) were split into multiple videos and individually analyzed using STICS. Each trial in figure 10 represents one recording of beads diffusing in solution that was split into 3 separate movies, each of which was then analyzed using STICS. The mean of the 3 movies for each trial is presented in figure 10 along with their standard deviation. Image MSD analysis was also done for each of the trials, and the resulting diffusion coefficients are plotted on figure 9 as well. Diffusion coefficients as measured by iMSD have a standard deviation of $1.38 \times 10^{-14} m^2/s$ across the 6 trials, whereas the standard deviation across trials as measured by STICS is $7.02 \times 10^{-15} m^2/s$. In contrast, the mean standard deviation of $D$ amongst movies within a given trial as measured by STICS is only $3.23 \times 10^{-14} m^2/s$. This indicates that the diffusion coefficient measured by STICS is consistent within a movie, but differs from iMSD measured $D$’s by a different amount across movies. In other words, within a given movie diffusion coefficients calculated by STICS appear to be off by a certain calibration factor, $\beta$, from iMSD values, but this factor changes for different movies.

IV. DISCUSSION

The ability of STICS to measure diffusion shows promise, but is yet to be fully validated. Its ability to measure a linear relationship between $1/D$ and $\eta$ in simulated datasets (figures 4 and 5) and the consistency with which it measures $D$ values for subsamples of a given experimental video lead us to believe that STICS is able to
measure diffusion, but needs to be calibrated by a cofactor $\beta$ that depends on the experimental setup. This $\beta$ takes the form of $D = \beta D_S$ where $D$ is the actual diffusion coefficient and $D_S$ is the value measured by STICS.

Through simulated datasets we have shown that when bead density and particle size are kept constant, STICS is able to measure the expected linear relationship between the viscosity of the solution, $\eta$, and the inverse diffusion coefficient, $1/D$. The linear region where this relationship holds necessitates sufficient movement of particles between frames. As we see in figure 4, the linear region for the 100 FPS video extends only to 0.1 Pa s, but when the video is down sampled to 25 FPS the linear region extends to 0.4 Pa s. With smaller changes between frames the Gaussians evolve less for a given $\tau$ value, and so the changes in peak width and location become harder to distinguish from noise – hence the extension of the linear region when the video is down sampled. With constant bead size and particle density, we see that $\beta$ remains constant, hence the linear relationship between $1/D$ and $\eta$. However, when certain conditional parameters change, such as particle density, $\beta$ also changes.

Because we are not able to trust the viscosities of the standards, in part due to uncertainties in temperature, we decided to validate the STICS measurements against those from iMSD. Furthermore, as shown in figure 6, MSD vs. $\tau$ plots of experimental videos show individual beads have differing rates of diffusion, a possible result of beads clumping in different amounts, especially at higher bead densities, and resulting in a range of effective radii. In figure 9 we see that for a given experimental setup with multiple videos taken of the same region, the measurements of $D$ by STICS differ from measured iMSD $D$ values by a constant factor $\beta$. However, between trials we see that $\beta$
changes, possibly attributed to localized differences in particle density or other factors that $\beta$ could depend on.

The origin of the calibration factor, $\beta$, is yet to be determined – its characterization and an understanding of its roots is the final hurdle in being able to measure diffusion using STICS. A large issue with characterizing the calibration factor lies in the creation of experimental standards with well-characterized parameters. These parameters include viscosity, particle radii, and temperature of the suspension. In future experiments the viscosity of standards with beads in suspension could be determined more precisely using cone and plate viscosity measures. Using beads with alternative coatings could reduce bead clumping and thus ensure constant particle radii. Lastly, ensuring the suspension equilibrates to the set temperature of the chamber on the microscope stage could control temperature. These parameters dictate diffusion rates, and when they are controlled we would expect variation in $D$ as measured by STICS to be due to changes in $\beta$, not the actual diffusion rate. Parameters of interest to consider that could affect $\beta$ are particle density in the region being imaged, which we already believe to play a role, signal strength, signal-bleaching rate, and imaging plane thickness.

A full characterization of $\beta$’s dependence on these parameters, and others it may depend on, would allow us to essentially calibrate STICS for a given experimental setup. That setup is not limited to beads diffusing in a suspension, but could easily be extended to observing intranuclear molecular flow and diffusion without the constraints of current measurement techniques, opening a novel door to study the physical landscape inside the nucleus.
V. Figures

Figure 1. (Left) Sample frame from simulated bead dataset with 100nm beads. (Right) Sample frame from simulated bead dataset with 10nm beads.

Figure 2. (Left) Sample frame from experimental bead dataset with 200nm beads at a 1% concentration. (Right) Sample frame from experimental bead dataset with 200nm beads at a 0.1% concentration.
Figure 3. Inverse of diffusion values measured by iMSD (red circles) for simulated datasets generally agrees with the linear prediction of Stokes-Einstein (blue line). Deviation observed from expected values is likely due to small sample size of beads that were successfully tracked.
Figure 4. Measurement of $1/D$ by STICS for simulated 100nm bead datasets shows an expected linear region in both the 100 FPS and 25 FPS simulations with only a 5% difference in their slope, but the linear region extends from 0.1 Pa s for 100 FPS to 0.4 Pa s for 25 FPS. However, both linear relations differ in their slope from the expected slope predicted by Stokes-Einstein by a calibration factor $\beta$. 
Figure 5. Measurement of 1/D by STICS for simulated 10nm particles shows a linear regime with respect to viscosity. Increasing the particle density in the simulation by 33% from 30 particles/um$^2$ to 40 particles/um$^2$ increases the value of $\beta$ by 25% from 0.0031 to 0.0039.
Figure 6. (Top) MSD vs. $\tau$ plot for the 3 beads identified in the experimental setup pictured in the bottom image. Varying rates of diffusion between the three beads could imply bead clustering, increasing the effective radius and thus decreasing the diffusion rate as seen in bead 3. (Bottom) Sample frame from experimental 100nm bead dataset in a 2M sucrose solution.
Figure 7. The $D$ values measured using iMSD across different parameters differ from the Stokes-Einstein theoretical predictions by varying amounts, potentially due to bead aggregation or inaccurate temperature and viscosity assumptions.
Figure 8. Due to the inability to calibrate STICS against theoretical values predicted by Stokes-Einstein, we calibrate the STICS measured $D$ (vertical axis) against the $D$ value measured by iMSD (horizontal axis). The resulting plot shows linear relationships for each size of bead, with $\beta = 0.075$, $\beta = 3.703$, and $\beta = 0.117$ for the 200nm, 100nm, and 20nm beads respectively.
Figure 9a. Three trials of experimental bead dataset videos (200nm beads in 2M sucrose at a 0.1% bead density) were subsampled and analyzed with STICS. The mean $D$ of the subsamples and their standard deviation for a given trial is plotted on the graphs, as is the $D$ value measured by iMSD for the trial.
Figure 9b. Three trials of experimental bead dataset videos (200nm beads in 2M sucrose at a 1% bead density) were subsampled and analyzed with STICS. The mean $D$ of the subsamples and their standard deviation for a given trial is plotted on the graphs, as is the $D$ value measured by iMSD for the trial.
VI. REFERENCES


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