Quantitative phase microscopy: automated background leveling techniques and smart temporal phase unwrapping

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In order for time-dynamic quantitative phase microscopy to yield meaningful data to scientists, raw phase measurements must be converted to sequential time series that are consistently phase unwrapped with minimal residual background shape. Beyond the initial phase unwrapping, additional steps must be taken to convert the phase to time-meaningful data sequences. This consists of two major operations both outlined in this paper and shown to operate robustly on biological datasets. An automated background leveling procedure is introduced that consistently removes background shape and minimizes mean background phase value fluctuations. By creating a background phase value that is stable over time, the phase values of features of interest can be examined as a function of time to draw biologically meaningful conclusions. Residual differences between sequential frames of data can be present due to inconsistent phase unwrapping, causing localized regions to have phase values at similar object locations inconsistently changed by large values between frames, not corresponding to physical changes in the sample being observed. This is overcome by introducing a new method, referred to as smart temporal unwrapping that temporally unwraps and filters the phase data such that small motion between frames is accounted for and phase data are unwrapped consistently between frames. The combination of these methods results in the creation of phase data that is stable over time by minimizing errors introduced within the processing of the raw data.

1. INTRODUCTION
The growing field of quantitative phase microscopy (QPM) allows for living semitransparent objects with very little inherent contrast such as cells to be quantitatively measured over time, nondestructively observing dynamic events within living systems not observable by traditional imaging such as brightfield, differential interference, and confocal microscopy [1]. However, to make the QPM measurements generally useful, significant data processing, including phase unwrapping, must be performed to convert a raw phase measurement into a meaningful sequence of data upon which biological conclusions can be extracted. In QPM, the measured phase correlates to the real quantity of optical path differences which can be linked to surface height deviations, optical thickness differences, and dry cell mass [2,3].

The initial data analysis step necessary in QPM, regardless of the imaging configuration employed (Linnik interference objective, digital holography, etc.), is the critical step of phase unwrapping. Phase unwrapping has been a topic of constant discussion since algorithm development first began in the 1980s [4,5]. New algorithm development in combination with new measurement techniques have paved the way for improved metrology solutions in a variety of application spaces. For this paper, techniques will be presented that are relevant to the field of QPM.

It was first suggested in the 1950s that interferometry could be applied to biological specimens to measure the phase of an object and relate that value to dry cell mass [2,3]. Since then, the field of QPM has rapidly grown and now encompasses many different techniques that allow for the phase of a biological specimen to be measured. However, to obtain measurements that accurately reflect an estimate of the actual object, there is significant processing of the raw measurement data that must occur. This paper will highlight many of the major important calculations and processing algorithms necessary to
measure biological objects in a dynamic environment accurately and consistently.

A quantitative phase microscope, 4D Technology’s BioCam, was developed to measure various biological specimens [6]. The majority of datasets generated by this system capture dynamic events where the phase of an object can be rapidly quantified during a critical biological process. These datasets are challenging to obtain and process for many reasons. Biological specimens, such as cells, are typically not well-behaved metrology targets. There is potentially significant motion of the objects of interest that occur between sequential frames of data. These cells sometimes become thick and densely spaced, creating phase regions with slopes much greater than the \( \pi \) radians slope restriction that interferometric measurements can reliably unwrap [7].

The work presented here shows a new method, smart temporal phase unwrapping that combines both spatial and temporal unwrapping in order to make phase sequences consistent in time. This method consists of a two-dimensional phase unwrapping routine in combination with a modified temporal unwrapping procedure that corrects for inconsistently unwrapped regions between two adjacent frames, even when there is slight motion. In addition, this technique allows for single frames where a local region is unwrapped differently amongst frames, to be regionally adjusted such that it is temporally consistent. The image processing routines discussed in this work serve to minimize errors within the phase unwrapping process, converting the raw measurements from the QPM into scientifically meaningful and consistent data. As the field of QPM continues to grow and in order for the resultant data to be a relevant scientific tool, errors within the phase retrieval process must be minimized.

2. METHOD

Quantitative phase measurements were made using a BioCam quantitative phase microscope (QPM) based on a Linnik configuration with a pixelated polarization phase mask [6]. This instrument utilizes phase-shifting interferometry to obtain multiple phase-shifted images of the interference between a reference mirror and an unknown sample measured in reflection. By utilizing a pixelated phase mask in front of the camera and appropriate polarization optics, four phase-shifted images are obtained simultaneously in one camera frame [8,9]. These four phase-shifted images can be used to calculate the wrapped phase, modulo \( 2\pi \) radians, of the measured object on a pixel by pixel basis using the four-bucket phase-shifting algorithm [10].

Obtaining the phase (modulo \( 2\pi \) radians) via an interferometric measurement is a very important step in obtaining unknown surface information. However, the resultant phase must be unwrapped such that it is no longer modulo \( 2\pi \) radians and represents an accurate and continuous estimate of the measured surface. There have been many reviews on the general subject of phase unwrapping [10,11] as well as analysis of how well unwrapping algorithms work on datasets for particular applications such as synthetic aperture radar [12] and Fourier transform profilometry [13]. A similar review of unwrapping methods applied to QPM data is beyond the scope of this work but would be a useful topic of discussion for the future as the number of researchers in this field continues to grow.

For the purposes of datasets presented in this work, a two-dimensional quality-guided path following the unwrapping algorithm using modulation or local slope as the quality metric was used [11]. When a two-dimensional phase unwrapping technique is applied individually to multiple frames of sequential data from the same changing object, the ability to accurately estimate the true phase of the measured object consistently over time can be diminished. The measurement data should be nominally the same depending on the process being observed. However, it is often the case that small features, the presence of noise, and potential small shifts due to vibration between measurements cause very different phase estimates on a frame-by-frame basis, as shown in Fig. 1.

This figure highlights two common problems with the unwrapped phase data: inconsistent phase unwrapping evidenced by the different heights calculated for sections of the data, such as the tip of the rotifer and residual background shape, in this example small amounts of residual tilt, due to a non-null interference measurement. Since each frame of presented data is unwrapped differently, which frame’s phase unwrapping results reflects the most accurate phase estimate? It is typically left to the observer to manually choose the image that appears to be the most realistic estimate and the erroneous measurements are not considered, causing missing data points. A non-null measurement configuration due to small tilts of the object with respect to the interferometer is a well-recognized problem present in nearly every interferometric measurement made [14]. The final phase measurement presented to a user should not include background shape such as tilt which is not a meaningful part of the dataset. The purpose of this paper is to present methods that provide automatic background shape removal and remove phase unwrapping inconsistencies in a dynamic dataset via smart temporal phase unwrapping.

3. AUTOMATIC BACKGROUND REMOVAL

The automated background leveling technique reduces background shape and decreases fluctuations in the mean value of the background for QPM datasets with large numbers of frames requiring little to no user-intervention. To remove the background shape, the object of interest must be separated from the background. Background shape within a phase dataset can be removed manually, as is the norm within the interferometric community, particularly for the measurement of objects such as steps. Manual removal is not an efficient method for datasets with hundreds of frames of data; however, it serves as a baseline means of comparison for exploring automated

![Frame 87](5.8 s) ![Frame 88](5.87 s) ![Frame 91](6.1 s) ![Frame 96](6.4 s)

Fig. 1. Phase measurement of a Rotifer showing inconsistent phase unwrapping between four frames of data.
techniques. As an example, manual background removal was performed on a phase measurement of human embryonic kidney cells (HEK293), as shown in Fig. 2.

The original unwrapped phase is shown in Fig. 2(A), while a manually selected background region is shown in Fig. 2(B). The best-fit plane fit to this region and extrapolated over the entire image is shown in Fig. 2(C), and the residual calculated surface is shown in Fig. 2(D). The background which was previously dominated by tilt now appears very nearly flat highlighting the topography of the cells without the bias introduced by the background tilt.

Manual background selection is exceedingly time-consuming on datasets with a large number of individual measurements. A user would have to manually adjust the background region throughout the sequence of data. This is too time-intensive to be feasible for general use. As a solution, an automated method has been developed and implemented that successfully operates on a broad range of objects, determines background pixels for each frame, and removes background shape without the need for user intervention. In order for the technique to be effective on many different QPM datasets, it must be very general and have built in redundancy to be able to differentiate objects from the background when the objects have varying spatial frequency content as well as different magnitudes of background variation such as tilt.

The first step in automatically removing background shape is separating an image’s content into background and object regions. This can be accomplished using the square of the phase gradient map (SPGM) which is a measure of the effective local slope within a single frame measurement. SPGMs are very sensitive to object boundaries and are commonly used for edge detection in classical imaging processing routines [15,16]. These SPGMs can be calculated as a function of the squares of the x- and y-phase gradients as is shown in Eq. (1) [17]:

\[
\text{SPGM} = \left( \frac{\partial \phi}{\partial x} \right)^2 + \left( \frac{\partial \phi}{\partial y} \right)^2.
\]  

(Note that our definition is actually the square of the phase gradient magnitudes, and that it is more sensitive to use this definition as it is more sensitive to changes in gradients and minimizes the effects of noise in phase maps.)

There are some critical assumptions that must be made in the development of an automated background leveling technique. The first is that the background can be, but is not restricted to, a small percentage of the total area. Additionally, larger SPGMs are associated with object pixels. Objects tend to have much greater and more widely varying local slopes than background regions. This is valid for cells and single-celled organisms. The final assumption is that larger phase values are associated with object pixels. This in general is always true when there is very little tilt present in a measurement and will be violated in the case of large tilt as will be shown in the following explanation. The background regions will appear to have smaller phase values than the objects of interest. The steps of the automated background leveling technique can now be presented in detail:

1. Calculate SPGMs and associate larger SPGMs with the object.
2. Associate larger phase values with the object.
3. Fill holes, i.e., regions that are completely surrounded by object pixels are also associated with the object. At this point, the object mask is now fully defined.
4. Calculate the background shape as the best-fit plane of the background pixels.
5. Calculate the residual surface by removing the background surface from the original phase data.

It is helpful to visualize the steps of this technique with an example of the HEK293 cells. For all binary masks shown, object pixels are displayed in white and background pixels are black. Initially, the SPGMs are calculated and larger values are associated with the object, as shown in Fig. 3(A). Large phase values are associated with the object, as shown in Figs. 3(B) and 3(C). The combination of these two operations successfully masks the boundary of the object in addition to masking off interior and flatter regions in the middle of a cell or organism. Notice that in Fig. 3(B), the white region at the top of the image corresponds to a region with large phase values due to the presence of background tilt. By iterating through the automated background leveling technique several times, the residual tilt can be decreased gradually and object pixels more accurately identified.

It is common to have flat regions that are unmasked in the middle of a cluster of cells. It is for this reason that the “holes” are filled in as is shown in Fig. 4. This is a key point to effectively mask objects from the background. Object boundaries must be continuously connected in order for this step to be effective.

The final step is to apply the final mask to the original unwrapped phase data and remove the best-fit surface. This process is summarized in Fig. 5 for the HEK293 cells as well as for a dataset showing a paramecium.

![Fig. 2.](image) HEK293 Cells with a manual background subtraction showing (A) the original unwrapped phase, (B) a background region manually selected, (C) the best-fit planar surface, and (D) the residual phase surface with the background shape removed.

![Fig. 3.](image) Steps 1 and 2 of the automated background leveling technique are illustrated including (A) SPGM calculations and masking large phase values in the first iteration (B) where regions with large tilt and subsequently large phase values are erroneously associated with object pixels. The second iteration (C) of the automated background leveling process no longer associates the top of the frame with object pixels.
By iterating this algorithm several times, the background mask is refined as the amount of background tilt is minimized. In this way, the background is more effectively selected and the automated background shape removal is successfully performed. With an automated technique established that effectively operates on datasets of both large single-celled organisms as well as cells, it is useful to quantify the performance of this technique over many frames of data.

A. Performance Comparison of the Automatic Background Leveling Technique

The performance of the automated background leveling technique was compared to a manual background removal for the examples of the HEK293 cells and paramecium datasets. It is important to validate that the automated technique performs well over many frames of data and yields consistent results.

The performance of the automated and manual background leveling techniques are quantifiably compared using two different metrics: residual tilt magnitude and mean background value variations within regions of known background. The tilt magnitude in the background region can be calculated using the Zernike tilt coefficients, $Z_1^1$ and $Z_{-1}^1$[18]. This is shown in Eq. (2):

$$\text{Tilt Magnitude} = \sqrt{(Z_1^1)^2 + (Z_{-1}^1)^2}.$$  (2)

For this set of tests, there are two background regions considered: Region A, used to perform the manual background leveling and Region B, used to test how effectively tilt was removed over that region. It is imperative that both regions consist for the majority of pixels associated with the background across all frames of data being considered. Any variation within these regions reflects the noise present in the measurement. These regions are shown below in Fig. 6.

In the presented analysis that follows, there are four test cases considered for each dataset. They are (1) the unwrapped phase without any background leveling shown in black, (2) an automatic background leveling procedure applied to each individual frame shown in blue, (3) an automatic background leveling procedure on the first frame then removing that surface from all other frames in the dataset while maintaining the same mean for the background shown in green, and (4) a manual background leveling using Region A to define the background shown in red. The calculated residual tilt magnitude for both datasets over a sequence of sequential frames is shown in Fig. 7.

Both datasets had a significant amount of tilt initially present. A manual background removal resulted in better correction than either of the automated methods. This is not surprising since the features of interest are complicated, especially for the case of the paramecium. One reason for this is because there is significant motion of the paramecium over the 20 frames of data considered as opposed to the HEK293 cells where there are only five frames of data and very little motion. Ideally, one would like the residual tilt magnitudes to be close to zero for all background removal cases, however, this is not
generally feasible. Due to the general nature of the automated background removal technique introduced, there are limitations to its ability to find and isolate background regions. It is meant to be a general technique that operates relatively well on a large set of potential cases to minimize the amount of user intervention necessary. The magnitude of the variations in Fig. 7 corresponding to uncertainties in the estimates of phase within the objects is determined in the next section.

B. Variation in Background Phase Values as a Background Leveling Performance Metric

For most measurements, there will be background fluctuations due to the fact that the media surrounding the cells and organisms of interest is not purely one solution of one singular refractive index. There are tiny particles that are barely resolvable, variations in refractive index, and other error sources that cause background fluctuations on a small scale with respect to the features of interest. These are ultimately the limiting error in QPM measurements. It is important to characterize the performance of the background removal techniques with respect to these background fluctuations. This can be accomplished by looking at the mean value of the phase in the known background area, Region B. The mean value should theoretically be zero after background shape removal.

The simplest universal method of characterizing the stability of the background removal techniques is to calculate the standard deviation of the mean phase values of background Region B for each dataset. This is displayed as the percentage of the maximum peak-valley phase of the original measurement. The background removal technique that results in the smallest standard deviation percentage over all frames has the greatest stability and will be the best technique to apply to future datasets. The standard deviation percentage values for the HEK293 cells and the paramecium are shown in Table 1.

The measurement that is most stable for all cases in the manual background removal, however, requiring a user to perform this task for large datasets is, in reality, unfeasible. It serves as an excellent point of comparison since it is by far the most stable technique. Implementing the automated background leveling on all frames of data or just the first frame in a set resulted in very similar performance. Using an automatic background leveling technique on each individual frame of a dataset can provide some benefit relative to the original unwrapped phase data. However, it does not seem necessary to create reliable zero mean data if the in-plane motion is small (less than 5% of full field).

Table 1. Stability of Background Removal Techniques over Background Region B^*

<table>
<thead>
<tr>
<th>Technique</th>
<th>HEK293 (%)</th>
<th>Paramecium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwrapped phase, no background removal</td>
<td>5.35</td>
<td>2.83</td>
</tr>
<tr>
<td>Background removed every frame</td>
<td>0.11</td>
<td>0.28</td>
</tr>
<tr>
<td>Background removed Frame 1 applied to all</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>Manual background removal</td>
<td>2.5E-13</td>
<td>0.1</td>
</tr>
</tbody>
</table>

^*% of the peak-valley phase value.

For future implementation, it is recommended that the background surface shape be characterized in the first frame of data and be subsequently removed from all other future frames of data to increase computation speed. It is necessary to adjust the mean value of the background of each individual frame relative to the first frame to ensure the best results. The automated background leveling method successfully handles the problem of removing the error sources within a measurement due to small variations in measurement conditions.

It is quite possible to adapt the automated background leveling technique such that it is better suited to treat either the HEK293 cells or the paramecium. However, creating one algorithm that removes background shape from general measurements is nontrivial. For some applications with two or three classes of objects, the algorithm could be easily customized to target those objects and remove the need for iteration within the algorithm, increasing computation speed. However, for the purposes of this work, a general algorithm that can be robustly applied to a wide variety of measurements is highly desirable and is thus the focus of the work.

4. SMART TEMPORAL UNWRAPPING

In fields outside of QPM, there have been suggestions on how to deal with inconsistent phase unwrapping of dynamic datasets [19–31]. When observing video-rate QPM data, there is often a distracting flickering of inconsistent phase values visible to the user. In addition to the distraction, it also causes significant errors in localized calculations of optical thickness over time. For all methods discussed, the assumption must be made that the phase change between any two pixels, either spatially or temporally adjacent, is less than π radians. For datasets with large amounts of motion (>10 pixels), the validity of this assumption comes into question. Another case that cannot be simply considered is that of an object entering and leaving the field of view. Features of this nature can be kept track of by counting the number of features present in an image, but this problem is outside the scope of this work.

The most straightforward technique to unwrap time-dependent datasets is temporal phase unwrapping. This has been widely applied to data collected using magnetic resonance techniques used to observe cardiac motion. For these datasets, temporal unwrapping via Itoh’s method is sufficient and has been implemented by several different researchers [19,20]. The first frame of data is used as a reference and the data are unwrapped in time on a pixel-by-pixel basis using one-dimensional phase unwrapping. One major disadvantage of this technique is the potential propagation of noise.

A more complex case occurs when the spatial and temporal phase information contains wrapped phase values while interframe motion stays minimal. It has been proved that unwrapping the phase of N-dimensional datasets is theoretically possible [21]. This has led to more theory exploring residue loops in three-dimensional space [22,23] and other three-dimensional phase unwrapping strategies [24].

Spatiotemporal phase unwrapping is a general term that refers to methods that reconcile motion between frames and unwrap the data either as a three-dimensional array or use temporal unwrapping methods to ensure the phase data are
self-consistent as a function of time. There are only a few algorithms that have been developed to process measurements in which there is significant interferometric motion and implement three-dimensional phase unwrapping [25–27]. These algorithms typically have several discrete steps: first applying a motion registration technique, then sequentially spatially and temporally unwrapping the phase data. This has been applied to the synthetic aperture radar deformation series to observe topographical changes to land masses [28–31]. Since this is a remote sensing technique, individual measurements are taken at different positions in orbit with varied look angles. The interferograms are resampled to account for these differences and minimize registration error. Once the look angle is accounted for, the interframe motion between adjacent pixels is assumed to have phase differences less than π radians and three-dimensional phase unwrapping techniques can be applied to the data.

The application of these existing techniques to QPM data provides several challenges and creates new potential sources of error. Temporal and three-dimensional phase unwrapping techniques, strictly speaking, assume no motion between datasets which is not generally a valid case. Additionally, the noise propagation that occurs due to the fact that individual frames of data are spatially wrapped makes these unattractive methods to pursue. Applying motion registration techniques to phase data is problematic for several reasons. The motion registration algorithm could be applied to the intensity data, wrapped phase, or unwrapped phase from a QPM measurement. Intensity data provides the most consistent data from frame to frame because it contains no height information which is subject to error from the unwrapping process. The features that can be tracked using intensity are limited to those that have enough inherent contrast. Wrapped phase and individual interferograms cannot be used because the mean value fluctuations between frames cause the point at which the data wraps to vary. The unwrapped phase is a suitable candidate for motion registration. However, the features that are inconsistent between frames may not register properly since they appear to be very different in sequential frames.

The biggest downside of using motion registration techniques is the necessity to resample the data on a new grid of points. This inherently limits the resolution of the resultant unwrapped phase data. Since losing spatial resolution is highly undesirable, motion registration techniques will not be considered at this time. For this paper, it will be shown that the inconsistent phase unwrapping problem can be solved using less involved techniques, namely, smart temporal unwrapping (STU). It is a simple approach based primarily on a modified temporal phase unwrapping with a filtering step and operates on data that has been previously unwrapped using a two-dimensional phase unwrapping algorithm.

A. Estimating Phase Unwrapping Accuracy

A critical component of STU is a tool that aids in estimating phase unwrapping accuracy. One potential starting point in the evaluation of phase unwrapping accuracy is determining the number of places where adjacent pixels in the unwrapped phase map have local slope values (differences between the phase of adjacent pixels) exceeding 2π radians. This has been suggested as an evaluation tool for use in Fourier transform profilometry as well as electronic speckle pattern interferometry [32,33]. Intuitively, this would appear to be an effective method; however, this method quickly breaks down and provides inconsistent results when looking at real datasets acquired using QPM. The deficiencies of this analysis method have never before been discussed in the literature.

As an example, a glass bead immersed in fluid was measured with a QPM and the data was unwrapped using both modulation and slope as the quality metric for quality-guided two-dimensional phase unwrapping, as shown in Fig. 8.

The glass bead’s overall spherical shape is unwrapped more accurately using modulation as a quality metric despite the large number of discontinuities seen along the top edge. The number of neighboring pixels whose slope difference is greater than 2π radians is 49 points for the modulation-based unwrapping and one point for the slope-based method. Using a count of remaining slopes greater than 2π radians within the unwrapped phase maps would indicate that Fig. 8(B) is the more accurately unwrapped phase estimate of the glass bead. However, there are clearly many sloped regions within the image that are greater than π but less than 2π radians. This is just one simple example where slope counting results in a misleading result.

To further illustrate the inconsistencies in the use of first derivatives, Fig. 2 shows the 1st, 2nd, and 3rd derivatives of

![Fig. 8. Comparison of phase unwrapping of the same dataset of a glass bead immersed in liquid using (A) modulation and (B) slope as the quality metric for two-dimensional phase unwrapping.](image)

![Fig. 9. 1st, 2nd, and 3rd derivatives of the glass bead’s unwrapped phase acquired using modulation and slope-based quality-guided phase unwrapping.](image)
The third derivatives can be used in the same way as the maps can also be assumed to be continuous if the surface being measured is continuous and second derivatives. Assuming continuity of the derivative maps alone do not provide enough information to draw conclusions about measurement accuracy. The information contained in the slope maps is not unique enough between unwrapping methods and does not isolate information that is related predominantly to unwrapping error.

Second derivative maps provide information related to curvature changes and are shown in the second row of Fig. 2. It is likely that extreme values in black and white correspond to regions that are being sampled below the Nyquist frequency. Features that are sufficiently sampled should exhibit smooth second derivatives. Borrowing from sub-Nyquist interferometry theory, if the surface being measured is continuous and sampled sufficiently, the second (and higher order) derivative maps can also be assumed to be continuous [34]. For the case of the glass bead, there are obvious problem areas around the edges of the glass bead. The second derivative maps of the slope-guided unwrapping show discontinuities along nearly the entire perimeter of the bead. The modulation-based unwrapping has a discontinuity along one edge, which is also emphasized in the second derivative map but is less pronounced than the slope-guided case. Whether the discontinuities in the derivative maps are due to unwrapping errors or undersampling, they can be used in either case to indicate unwrapping accuracy estimates.

The same differences between the two unwrapping methods can be seen in the third derivatives, as shown in the third row of Fig. 2. Third derivatives can be used in the same way as the second derivatives, assuming continuity of the derivative maps as an indicator of superior phase unwrapping.

Considering either the second or third derivative maps, the majority of points are centered about a mean value. The outliers are points that are due to slope discontinuities connected with poor phase unwrapping. If the frequency of outliers of the two unwrapping algorithms is compared, it is expected that the more accurate unwrapping algorithm will be the one with fewer outliers away from the mean, i.e., the smoother high-order derivative map corresponds to the more accurate unwrapped phase map.

To perform this test, the same level derivative from each unwrapping method is compared via their histograms. Since the majority of data points lie about the mean value, only the outlier information needs to be considered. A threshold is automatically determined that corresponds to the mean separation between peaks in the histograms. This is a simple, fast, but approximate estimate of the location of the minimum location between peaks. It is an approximate method since not all datasets considered have well-defined zero points between the maximum and side lobes. The same threshold value is applied to both sets of the histogram data. The histograms of the second derivation in the y-direction are shown below in Fig. 10.

This method was shown in practice to be universally consistent for a wide range of QPM data. The examples in this section show how using higher order derivative outliers can be used to predict accuracy of phase unwrapping between two methods.

B. Smart Temporal Unwrapping Algorithm
The STU method will be applied to several different cases where unwrapping inconsistencies are both small and large percentages of the field of view. The real problem with the inconsistencies in the phase unwrapping is that they can cause drastically inconsistent quantitative analyses over the course of a measurement. This may be caused by the steep edges of cells and single-celled organisms that are hard to successfully unwrap likely due to the fact that they are violating the assumption that all local slopes must be less than π radians.

A smaller local region that displays phase unwrapping inconsistencies within the rotifer tail is shown in Fig. 11. In this case, there are small inconsistent regions that flash dark blue to light blue as a function of time (circled in black). These examples show the variety of small and large features that have erroneous two-dimensional phase unwrapping estimates.

A smart temporal phase unwrapping method (STU) has been created to locally correct for phase unwrapping inconsistencies as a function of time. The algorithm consists of two main steps: a localized temporal phase unwrapping and a filtering step. For the explanation that follows, two frames of phase data, A and B will be considered. Frame A is the initial frame and is assumed to be the best individual frame of data within the set based on the arguments presented in the previous section. The complete unwrapping process is as follows:

- Two-dimensional phase unwrapping: Unwrap Frames A and B using two-dimensional phase unwrapping.

![Fig. 10. Histograms of the Dy2 data for slope- and modulation-based unwrapping methods. The cutoff values for both are calculated and displayed in red on the histograms. It is evident that the slope-based unwrapping method has many more outliers outside of the main peak.](image)

![Fig. 11. Inconsistent phase unwrapping is shown in regions of the zoomed-in rotifer tail. The regions that change as a function of time are circled in black.](image)
• Background leveling: Remove the background shape of both frames using the automated leveling routine discussion in the previous section.

• STU, part 1: Temporally unwrap Frame B with respect to Frame A. Only temporally unwrap inconsistent regions of size 15 pixels or more. This avoids temporally unwrapping singular noisy pixels. Note that this step does not account for any image motion.

• STU, part 2: Filter the temporal unwrapped Frame B to account for artifacts due to motion. Test the region using continuity of derivatives to determine whether or not temporal unwrapping provides a better local solution.

This procedure can then be repeated for all subsequent frames of data, replacing Frame A with Frame B’s unwrapped phase values until all data have been processed. With the initial steps laid out, each step will be discussed in finer detail.

Data processing begins by two-dimensionally phase unwrapping Frames A and B and removing the background shape and mean value fluctuations using the automatic background removal technique addressed in Section 3. The results for Frames 1 and 6 of the rotifer tail dataset are shown in Fig. 12. Nonadjacent frames of data were chosen for this example to show the effects of greater motion, although it is still minimal for this example (~6 pixels).

In this case, the differences between Frames A and B are subtle, highlighted in black, these regions have slightly varying phase values near the boundaries of the rotifer body. The STU scheme can now be applied to the data. The first step is to temporally unwrap Frame B with respect to Frame A. The two frames are shown next to each other after performing a temporal phase unwrapping operation as seen in Fig. 13.

Once again, the differences are subtle, so the undesirable artifacts are circled in black. The temporal unwrapping does not account for motion, so it is quite likely that regions where motion has occurred create unrealistic phase jumps. By filtering the data, local regions are tested to see if the temporally unwrapped phase is really an improvement upon the original two-dimensional phase unwrapping. To begin, regions need to be identified that exhibit unwrapping differences in Frame B between the original unwrapped phase and the temporally unwrapped version.

Identifying the local regions to test the validity of the temporal unwrapping is a critical step. A very obvious choice would be to isolate all pixels whose phase values were changed during the temporal unwrapping. For the datasets shown, the temporal unwrapping step is applied to groups of 15 pixels or greater that exhibit changes between sequential frames. If that group size is increased to a larger value, for example 10% of the array size, only large features with inconsistent phase unwrapping will be changed using a temporal unwrapping. These large features are less likely to move distances greater than their own size between two subsequent frames and will therefore likely be temporally unwrapped accurately. Therefore, the regions that need to have their temporal unwrapping questioned are the small isolated regions within the image, not the larger regions. These smaller regions are more likely to have artifacts created by the temporal unwrapping due to motion between the frames that need to be accounted for and filtered appropriately. One simple way to find the regions that need to be analyzed and filtered is by considering the difference of the SPGMs between the original and temporally unwrapped versions of Frame B. A mask of data to be tested can be created by thresholding the difference data. Any difference value that is outside the mean by greater than one standard deviation will be tested. This effectively isolates regions where the temporal phase unwrapping has caused considerable changes in the local slope. For the example of the rotifer, the SPGMs can be calculated and the mask based on their differences created. It is shown below in Fig. 14.

Once the SPGM mask has been created, each individual region can be analyzed and tested to see if the temporally unwrapped results are superior to the original two-dimensional phase unwrapping. The continuity of derivatives test used previously introduced is implemented to test for phase unwrapping accuracy. Each isolated region within the SPGM mask

Fig. 12. Results for Frames A and B after going through step 1, two-dimensional phase unwrapping, and step 2, background leveling. Notice that in Frame B, there are small regions that have regions where the phase unwrapping appears to be different from Frame A, circled in black.

Fig. 13. Temporal unwrapping of Frame B with respect to Frame A. Notice the regions in black that are circled. Within these regions are pixels that, due to motion, are temporally unwrapped incorrectly.

Fig. 14. Temporally unwrapped frame is compared to the original phase unwrapping and a mask is created that highlights regions where the SPGM varies more than one standard deviation within the mean of the differences.
is individually considered. The mask is grown to incorporate nearest neighbors to create a more accurate test of the local region. The mask is applied to both the original and temporally unwrapped Frame B and the continuity of derivatives are tested. If the temporal unwrapping creates more discontinuities than it resolves, the temporal phase unwrapping is undone in that region. In this manner, all closed regions within the SPGM mask are addressed and the unwrapping in each local region is tested. This is shown visually in Fig. 15.

With a final correction mask now determined, the original two-dimensional phase unwrapping is only applied to masked regions, whereas the rest of the frame is the result of the temporal unwrapping. A summary of the STU applied to this dataset is presented in Fig. 16. This example shows that STU can correct for phase unwrapping errors that occur in the temporal domain.

A more interesting and obvious example is that of later frames of the same rotifer tail dataset. The phase unwrapping results vary wildly in large regions of the image. These frames of data become usable once STU is applied, as shown in Fig. 17. Another successful implementation of the STU scheme is shown in Fig. 18 of the zoomed-in region of the rotifer tail.

The STU algorithm successfully provides continuity across the frames of data. The data are made to be more consistent, while frames that contain significant motion are treated appropriately and overall phase unwrapping appears to be creating accurate estimates of any shape changes consistently over time.

C. Processed Data Example: Cardiac Myocytes

One example where QPM measurements processed with the STU algorithm have yielded novel scientific observations has been with the use of rat cardiac myocytes under the influence of different drugs [35]. These cells spontaneously beat on their own, much like our hearts, but without electrical stimulation. By observing these cells using a QPM and appropriate data processing applied, the strength and frequency of the beating can be quantified. Without background shape removal and application of STU, the data appears nearly random. Two still images are shown in Fig. 19 of the rat cardiac myocytes before and after treatment with isoproterenol hydrochloride (IPHC), a drug that increases beating frequency. It was observed that when the drug IPHC is applied to the cells, the beating frequency increases by a factor of eight, and the strength increases by a factor of three, as shown in Fig. 20.
Determining that the strength changes with IPHC application is not something that can be easily accomplished using conventional microscope techniques. By observing relative changes of the integrated optical volume of a fixed region as a function of time, the beating was quantified and characterized in a way that has never before been possible. QPM allows scientists to characterize the behavior of cells using relative measurements of optical thickness.

The beating rat cardiac myocytes is an excellent example of processed results from a quantitative phase microscope. The image processing is what makes these results reliable and accurate, eliminating sources of error within the measurement and phase unwrapping. These datasets become useful after the results are forced to be consistent in time and unwrapping errors and background fluctuations are minimized.

5. CONCLUSION

The STU method introduced in this paper operates successfully on several different kinds of biological datasets acquired with QPM. The purpose of this routine is to generally fix inconsistent phase unwrapping within a sequence of phase images by combining the two-dimensional phase unwrapping with automated background leveling and the STU method. By going through these steps, the phase data becomes much more useful since relative changes can be observed once all frames of data are unwrapped as similarly as possible.

This processing of the phase data has been shown to be effective for a broad variety of biological datasets. However, the STU method presented has some inherent limitations. Because there is no formal motion registration performed, assumptions have to be made about the size of regions that are allowed to vary temporally. For the implementation presented here, all groups containing 15 pixels or more that display inconsistent phase unwrapping are temporally unwrapped. The ability to accurately determine whether or not the application of temporal unwrapping to these smaller groups is necessary is dependent on the accuracy of the continuity of the derivative test. Generally, the continuity of the derivative test appears to be largely consistent with the qualitative results of a trained observer choosing their preferred unwrapped frame.

The ideas and algorithms presented in this article fully characterize the necessary steps to make QPM data useful in both the case of an individual frame of data as well as over the course of a dynamic measurement with large numbers of frames. Without this work, performing time-scale studies of biological specimens with QPM are likely to be error-prone. The elimination of local phase fluctuations due to phase unwrapping is an essential step in being able to conduct analyses that require consistent and accurate phase information. The methods explained and examples of datasets have shown that STU forces consistent temporal phase unwrapping, allowing a consistent estimate of the phase of biological objects to be made.

REFERENCES


