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Wavelet-based tracking of bacteria in unreconstructed off-axis holograms

Zach Marin¹, J. Kent Wallace^{2,3}, Jay Nadeau², Andre Khalil^{1*}

¹CompuMAINE Laboratory, Department of Mathematics & Statistics, University of Maine, Orono, Maine 04469

²The Motility Group, Division of Aerospace Engineering, California Institute of Technology, 1200 E. California Blvd., Pasadena CA 91125, USA

³Jet Propulsion Laboratory, California Institute of Technology, 4800 Oak Grove Dr., Pasadena, CA. 91109, USA

zachary.connertymarin@maine.edu, james.k.wallace@jpl.nasa.gov, jnadeau@caltech.edu, andre.khalil@maine.edu

*corresponding author

Abstract

We propose an automated wavelet-based method of tracking particles in unreconstructed off-axis holograms to provide rough estimates of the presence of motion and particle trajectories in digital holographic microscopy (DHM) time series. The wavelet transform modulus maxima segmentation method is adapted and tailored to extract Airy-like diffraction disks, which represent bacteria, from DHM time series. In this exploratory analysis, the method shows potential for estimating bacterial tracks in low-particle-density time series, based on a preliminary analysis of both living and dead *Serratia marcescens*, and for rapidly providing a single-bit answer to whether a sample chamber contains living or dead microbes or is empty.

Keywords

Wavelet transform; WTMM method; Airy disks; particle tracking; motility; off-axis holograms

1. Introduction

Digital holographic microscopy (DHM) is increasingly the imaging methodology of choice for particle tracking techniques as it can capture both amplitude and phase information of a 3D volume in a single shot [1-6]. Particles with low amplitude contrast can be detected via shifts in refractive index using phase information [1]. DHM focusing is done post-capture in a numerical reconstruction process [1, 2]. Thus, particle tracking can be done in a time series of holograms, optionally reconstructed at each time point, and the reconstructed images can be stacked together to create a 3D movie [5]. However, reconstructed image stack data is voluminous and reconstruction can be time consuming. Although some methods have been proposed to analyze unreconstructed holographic data, they were restricted to inline holography [7, 8]. This exploratory paper proposes an automated, wavelet-based method of tracking bacteria in unreconstructed holograms with the purpose of providing estimates of the presence of motion and particle trajectories in hologram time series.

Holographic particle tracking is commonly done with inline holography [8-10]. However, inline holograms must be taken at two or more time points, wavelengths, or camera-to-sample distances to allow quantitative phase shift measurements [1, 5]. Off-axis holography can instantaneously capture both amplitude and phase information due to the phase shift created by the angle between beams incident on a sample [1, 5]. As such, off-axis DHM can be used to create real-time 3D tracks of swimming bacteria.

Out of focus particles in a hologram are commonly found by matching characteristic light scattering simulations to real data [8] or by segmentation of particles via edge detection in reconstructed planes [9-11]. Matching real light scattering via Lorenz-Mie and Rayleigh-Sommerfeld scattering approximations provides depth information [6, 8, 12, 13]. A low-order approximation of this scattering, such as an Airy-like pattern, is less accurate but computationally more efficient. A phase particle, such as a transparent bacterium, is considered in focus when the amplitude of the particle in a holographic reconstruction is maximal [14]. As such, investigating gradients across reconstructed depths can reveal a particle's focal plane [11].

The off-axis holograms used in this paper were taken with an instrument developed specifically for imaging prokaryotes [5]. This device eliminates noise due to mechanical and light instabilities, which is necessary for field imaging in harsh conditions [3, 5]. However, speckle noise is inherent to the source coherence required to create high-contrast fringes across the array, and stabilization issues in imaging can introduce inconsistencies in amplitude across the image. Thus, image processing of off-axis holograms must account for the presence of speckle and traveling waves.

Multiresolution analysis techniques can use wavelets to examine the behavior of a signal across time or size scales, thus creating resilience to noise [15]. The 2D wavelet transform modulus

maxima (WTMM) method combines wavelet partial derivative information across scales to enhance resilience to intensity variation at both large (traveling waves) and small (speckle noise) scale [16-31].

The WTMM segmentation method is versatile in extracting important features hidden in a noisy background, from multiple imaging modalities. It was used as a segmentation procedure in fluorescence microscopy to analyze the position and morphology of chromosome territories in 2D projections of mouse lymphocytes [17, 23], to identify the boundaries of cell nuclei in 3D from fluorescence microscopy images of *Caenorhabditis elegans* embryos [20], to characterize the location and length of coronal loops from ultra-violet images of the sun [25], to perform a multifractal-based segmentation of solar photospheric magnetic structures using solar magnetogram data [21], to segment and characterize the morphology of ultra-thin gold surface from atomic force microscopy [26], and to detect microcalcification clusters hidden in breast tissue (x-ray) mammograms, and discriminate between their pathology (benign vs. malignant) via their fractal signature [16].

In this paper, the 2D WTMM segmentation method [16, 17, 20, 21, 23, 25, 26] with an isotropic 2D Gaussian smoothing function is used to track both living and dead *Serratia marcescens* in unreconstructed off-axis holograms. The WTMM segmentation method distinguishes unobscured Airy-like disks, created by light passing through the bacteria, from background in time differences of unreconstructed digital hologram time series. Calculated positions of individual *S. marcescens* are linked through time using the Hungarian algorithm [32] coupled with a radial nearest neighbors search, which accounts for multi-frame missed detections. The WTMM segmentation method is also applied to a time series containing no bacteria. Presence of tracks is used to distinguish empty from non-empty series. Track anisotropy is used to distinguish motile (living) from drifting (dead) bacteria.

2. Mathematical background

The 2D wavelet transform modulus maxima (WTMM) method [16-31] is a powerful multifractal formalism. The WTMM method utilizes a collection of continuous wavelet transforms (WTs) [15] as a mathematical microscope to examine the behavior of an image over a range of spatial frequencies, or so-called size scales in real space.

Let $\phi(x, y)$ be an isotropic 2D function [28]. Let

$$\psi_1(x, y) = \frac{\partial \phi(x, y)}{\partial x}, \quad \psi_2(x, y) = \frac{\partial \phi(x, y)}{\partial y}$$

be wavelets satisfying the admissibility condition (ψ_1 and ψ_2 have a zero mean) [15]. The WT with respect to ψ_1 and ψ_2 is

$$\mathbf{T}_\psi[f](\mathbf{b}, a) = \begin{pmatrix} T_{\psi_1}[f] \\ T_{\psi_2}[f] \end{pmatrix} = \begin{pmatrix} a^{-2} \int \overline{\psi_1(a^{-1}(\mathbf{x}-\mathbf{b}))} f(\mathbf{x}) d^2\mathbf{x} \\ a^{-2} \int \overline{\psi_2(a^{-1}(\mathbf{x}-\mathbf{b}))} f(\mathbf{x}) d^2\mathbf{x} \end{pmatrix} = \nabla(\mathbf{T}_\phi[f](\mathbf{b}, a)).$$

The WT can be expressed as the modulus and the argument

$$\mathbf{T}_\psi[f](\mathbf{b}, a) = (M_\psi[f](\mathbf{b}, a), A_\psi[f](\mathbf{b}, a))$$

where

$$M_\psi[f](\mathbf{b}, a) = \sqrt{(T_{\psi_1}[f](\mathbf{b}, a))^2 + (T_{\psi_2}[f](\mathbf{b}, a))^2}$$

and

$$A_\psi[f](\mathbf{b}, a) = \text{Arg}(T_{\psi_1}[f](\mathbf{b}, a) + iT_{\psi_2}[f](\mathbf{b}, a)).$$

WTMM are defined as the locations $\mathbf{b}$ where $M_\psi[f](\mathbf{b}, a)$ is locally maximum in the direction of the argument $A_\psi[f](\mathbf{b}, a)$ at scale $a > 0$. WTMM are grouped in connected chains [25] called maxima chains [28, 30, 33, 34]. The algorithmic details of chaining can be found in the Appendix of [25]. The WTMM method was implemented using CUDA on an NVIDIA Titan X Pascal. WTMM chains forming around an Airy-like disk in an image with background noise at small, medium, and large scale are shown in Fig. 1.

Fig. 1. WTMM chains forming around an Airy-like pattern in an image with background noise at small (left), medium (center), and large (right) scale. Note that the Airy pattern's central lobe, the Airy disk, is not caught at large scale. To ensure the correct chain is found, i.e. a chain at a small or medium scale, chains are thresholded on mean modulus as described in Section 3.3.

3. Materials and methods

3.1. Sample preparation and acquisition

The test strain used for tracking was *Serratia marcescens* ATCC13880 (American Type Culture Collection), which was grown overnight at a temperature of 30° C in nutrient broth (NB, Becton Dickinson) to mid-log phase. *S. marcescens*, a rod-shaped, gram-negative bacterium, was chosen as the test strain because of its characteristic bright red colonies, which allow for confirmation after recording that the samples were not contaminated. Several minutes before

recording, the mid-log cultures were diluted 10^4 - 10^6 fold in motility medium (10 mM potassium phosphate, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM glucose, pH 7.0) and pipetted into a 1.5 mm deep silicone sample chamber with an optical glass bottom (Electron Microscopy Sciences). The sample was covered with a second coverslip and imaged immediately. Dead bacterial controls were prepared by exposing *S. marcescens* to 70% ethanol for several minutes, then sedimenting by centrifugation in a microcentrifuge and resuspending in motility medium. An empty chamber control contained motility medium only.

The instrument used for hologram acquisition was an off-axis digital holographic microscope (DHM) developed specifically for imaging prokaryotes [5]. The optical specifications of this instrument are given in Table 1.

Table 1. Specifications of the Off-Axis DHM Instrument Used

Property [unit]	Value
Objective numerical aperture	0.3
Magnification	19.7
Illumination wavelength [nm]	405
Field of view [$\mu\text{m} \cdot \mu\text{m}$]	$360 \cdot 360$
Depth of field [μm]	1500
Lateral resolution [μm]	0.7
Camera pixel size [$\mu\text{m} \cdot \mu\text{m}$]	$3.45 \cdot 3.45$

Data acquisition from the DHM instrument was performed using the software Koala (LynceeTec). Live *S. marcescens* were tracked over ~3 minutes in intervals of ~170 milliseconds, resulting in ~1000 time points. Dead *S. marcescens* were tracked over another ~3 minutes in intervals of ~180 milliseconds, resulting in slightly less than 1000 time points.

3.2. Preprocessing

Wavelets work best on raw data [15], and as such preprocessing is kept to a minimum. However, speckle noise is lessened by adding or subtracting images of the same subject [35]. To remove noise that may interfere with isotropic wavelets, each hologram time series was recast as a series of image differences between successive time points. The difference is asymmetric rather than absolute, ensuring that information about Airy-like disks in the first frame is kept, while the second frame acts as a background approximation. A single time difference for two time points is shown in Fig. 2.

Fig. 2. A single time difference in a hologram time series. The image difference is calculated for every pair of consecutive time points $t1$ and $t2$ in the time series. Researchers could distinguish bacterial locations manually by searching for motion. Airy-like patterns are visible in the image differences, but not in the raw data.

3.3. Bacteria selection

Bacteria are selected from holograms using WTMM segmentation of Airy-like disks via Gaussian wavelet approximation over 80 scales. At each scale, WTMM are chained as described in Section 2. Chains are considered *closed* if the first and last points of their parametrization share the same position.

Let $\mathcal{C}$ be a closed chain. Each $C \in \mathcal{C}$ is a WTMM and is represented as a tuple $C = (M, A, \mathbf{b}, a)$, where $M, A, \mathbf{b}, a$ are modulus, argument, position, and scale, as defined in Section 2. To perform WTMM segmentation [17, 20, 21, 23, 25], the total set of closed chains is pared down via mean radius, filament index, and mean modulus of individual closed chains. For a closed chain C , let the boundary $\gamma = \{\pi_3(C) | C \in \mathcal{C}\}$, where $\pi_3(C)$ is the third projection mapping of C , i.e. $\pi_3(C) = \mathbf{b}$. Each element $\mathbf{b} \in \gamma$ can be parameterized as $\mathbf{r}(u) = (x(u), y(u))$ where $(x(u), y(u)) \in \mathbb{R}^2$ for all u . The perimeter P_C is defined as

$$P_C = \int_{\gamma} \sqrt{x'(t)^2 + y'(t)^2} dt.$$

The mean radius of $\mathcal{C}$ is

$$\overline{r_C} = \frac{P_C}{2\pi}.$$

Let $\mathbf{B} \subseteq \mathbb{R}^2$ be the image region circumscribed by C . The filament index [26, 35, 36] of $\mathcal{C}$ is

$$F_C = \frac{P_C D_C}{4A_C},$$

where D_C is the diameter, defined as

$$D_C = \sup_{(x,y) \in \mathbf{B}} \left(\sup_{(x',y') \in \mathbf{B}} d((x,y), (x',y')) \right),$$

where d is the Euclidean metric, and

$$A_{\mathcal{C}} = \iint_{\mathbf{B}} dx dy$$

is the area of $\mathbf{B}$. The filament index is a measure of $\mathcal{C}$'s circularity, with $F_{\mathcal{C}} = 1$ indicating a perfect circle and any value > 1 quantifying departure from a circular shape. The mean modulus of the chain is

$$\overline{M}_{\psi, \mathcal{C}} = \frac{1}{|\mathcal{C}|} \sum_{C \in \mathcal{C}} \pi_1(C),$$

where $|\mathcal{C}|$ is the cardinality of $\mathcal{C}$ and $\pi_1(\mathcal{C})$ is the first projection mapping of $\mathcal{C}$, i.e. $\pi_1(\mathcal{C}) = M$.

Chains are thresholded on radius $\overline{r}_{\mathcal{C}}$ and filament index $F_{\mathcal{C}}$ and clustered across scales by centroid as shown in Fig. 3. Clusters containing chains at 3 or more scales were considered indicative of an Airy-like pattern and kept as in Fig. 3, middle. In each cluster, the chain with the highest mean modulus $\overline{M}_{\psi, \mathcal{C}}$ was selected as the best approximation of the Airy-like disk as shown in Fig. 3, right.

Fig. 3. Selection of closed WTMM chains corresponding to Airy-like patterns in a hologram. (Left) All closed chains across 80 scales with $\overline{r}_{\mathcal{C}} > 3.5 \mu m$. (Middle) Remaining closed chains after thresholding $F_{\mathcal{C}} \leq 1.3$ and clustering across scales by centroid. (Right) Selection of highest mean modulus chain from each cluster. These threshold values were determined to be optimal via empirical results.

3.4. Depth Estimation

A phase particle is considered in focus when the amplitude of the particle in a holographic reconstruction is maximal [14]. When the particle's amplitude is at a maximum or a minimum, the gradient between the particle and the image background will also be at a maximum and therefore the mean modulus of a closed WTMM chain will be maximal.

As a particle moves away from the recording plane the Airy pattern dilates [13]. The width of the central lobe or Airy disk in a particle K 's Airy pattern is

$$d_K = 1.22 \frac{\lambda z_K}{\omega_K},$$

where λ is the wavelength of incident light, z_K is the distance from the particle K to the focal plane, and ω_K is the width of the particle [36]. Thus, assuming roughly uniform particle size, the Airy pattern of a particle far from the focal plane will be detected at a larger radius than a close particle. This equation is used to estimate depths of particles found in unreconstructed holograms. This depth estimation, however rudimentary, experimentally improved track estimation.

3.5. Track Generation by Hungarian Algorithm and Nearest Neighbors Search

The Hungarian algorithm [32] was used to solve the assignment problem between each successive pair of time frames. This algorithm minimized the sum of the edge weights in a directed, bipartite graph where time frames were represented by vertex sets and each vertex in each set represented a particle found at that time point. The Euclidean distance between particles at successive time points, with z-coordinates determined as described in section 3.4, was used to determine edge weights. A radial nearest neighbors search complemented the Hungarian algorithm. This search allowed separate tracks to be linked if the beginning of one track was within N pixels of the end of another track, and if each of these tracks ended within T time frames of one another. Simple Tracker by J.Y. Tinevez [37] performed both the Hungarian algorithm and the radial nearest neighbors search and was used to create tracks.

Let $\{T_l\}_{l \in L}$ be the set of generated tracks, where L is the number of generated tracks. Each track T_l is a set of coordinates $\{(x_{l,i}, y_{l,i}, z_{l,i})\}_{i \in \{1, \dots, |T_l|\}}$. Each track was thresholded on cardinality $|T_l|$ and mean displacement

$$\overline{T_{l,\sigma}} = \frac{1}{|T_l|} \sum_{i \in \{1, \dots, |T_l|\}} \sqrt{(x_{l,i} - x_{l,i+1})^2 + (y_{l,i} - y_{l,i+1})^2 + (z_{l,i} - z_{l,i+1})^2}.$$

4. Results and discussion

4.1. Distinguishing live from dead *S. marcescens*

Live and dead *S. marcescens* time series were acquired as described in Section 3.1. *S. marcescens* locations within time differences were calculated as described in Section 3.3 with $\bar{r}_c > 2.62 \mu\text{m}$ and $F_c \leq 1.3$, threshold values determined to be optimal based on empirical results. Tracks were generated from these locations in the time series as described in Section 3.5 and then linked into a single track if track endpoints were within $N = 13.13 \mu\text{m}$ and $T = 5$ time points of each other. Tracks were kept if $|T_l| > 5$ and $\overline{T_{l,\sigma}} > 1.75 \mu\text{m}$. These threshold

values were also derived empirically. Analysis of live *S. marcescens* yielded 205 tracks, while dead yielded 139.

A knowledgeable user manually tracked the centroids of Airy disks in the live and the dead *S. marcescens* time series based on visual inspection. The user found 64 tracks for live and 887 tracks for dead *S. marcescens*. A comparison of these user-labeled tracks (black) and the computer-generated, WTMM-based tracks (colors) is shown in Fig. 4. A video of time differences of the live *S. marcescens* time series overlaid with WTMM-based tracks is available online (Fig. S1).

Fig. 4. A comparison of the user-labeled live (top) and dead (bottom) *S. marcescens* tracks (black) and WTMM-based tracks (colors). Color indicates time step at which a point is found in the time series.

Dead *S. marcescens* had a net preferential direction, as opposed to live bacteria that swam freely in multiple directions, as shown in Fig. 5. The source of this preferential direction for the dead bacteria is likely drift due to mechanical vibrations, perhaps from placing the sample in the chamber and imaging too shortly after or from knocking the instrument. The live *S. marcescens* have an almost flat histogram. Thus, their motion is close to isotropic. It is likely that if there was drift in the dead sample, the live *S. marcescens* also experienced drift, but could counteract its effects by swimming against the flow direction. Further studies with larger statistical numbers could help infer whether the live bacteria consistently show no preferential direction and if they exhibit Brownian motion. If statistical significance is established, this can

be used to provide a single-bit answer—“Live” tracks or “Dead” tracks—to whether bacteria in a sample chamber are live or dead.

Fig. 5. Normalized histograms of swimming direction angles of live (red) and dead (blue) *S. marcescens*. All angles were relative to the x-axis. The dashed line represents the theoretical histogram for non-preferred swimming directions (pure isotropy).

4.2. Identifying empty sample chambers

An empty sample chamber was imaged over ~ 150 time points. The method described in Section 3.5 attempted to generate tracks using the same parameters as in Section 4.1. Tracks were kept if $|T_l| > 5$ and $\overline{T_{l,\sigma}} > 1.75 \mu\text{m}$. After this thresholding on track length and displacement, no tracks remained. Thus, empty chambers can be distinguished from chambers containing dead bacteria simply based on number of tracks. This is quite clear since even dead bacteria will be found repeatedly at or near the same location and linked, whereas only noise can be found in empty chambers. Further studies to show the lack of tracks is statistically common in empty chambers would allow for single-bit discrimination of empty chambers.

4.3. Validation of depth estimation

A hologram simulation was generated of a sphere with diameter $\omega_K = 1.75 \mu\text{m}$ moving in an arc as $y = 179.32(1 - \sin \frac{x}{358.65})$ where x shifted $17.5 \mu\text{m}$ and moved $20 \mu\text{m}$ in depth over 20 time frames. Holograms were simulated using angular spectrum propagation [1, 38] following the optical properties of the aforementioned DHM scope [5]. Clusters as defined in Section 3.3 were kept if $\bar{r}_c > 3.5 \mu\text{m}$, $F_c \leq 1.15$, and chains were found at > 2 scales in the cluster. These values were derived empirically. No noise was added to the simulation.

Fig. 6 shows tracking results of depth vs. the x-direction. Note that some depths were over- or underestimated due to WTMM chains sometimes forming in the second or third minima of the Airy pattern. In this simulation, the intensity per unit area ratios of Airy-like minima and maxima near the center of the pattern were such that the maxima contributed more energy than the minima canceled when the pattern was convolved with a wavelet. The generated track therefore did not span a full 20 μm in depth, although the track exhibits the expected trend of increasing depth.

Fig. 6. Results of depth vs. x-direction for particle moving in an arc. Color indicates time step at which a point is found in the time series. Note that the general trend is correct, but as the depth increases the estimation gets worse.

5. Conclusions

Wavelet-based particle tracking in unreconstructed off-axis holograms outlined in this paper can quickly search many sets of time-series holograms for motion, and successfully extract some bacterial tracks from a volume with low depth estimate accuracy.

The WTMM segmentation method accurately extracts centroids of unobscured Airy-like disks. These Airy-like disks are the first lobe of Airy patterns representing bacteria. The centroid can be used in scattering approximations. Accurate scattering fits at Airy-like centroids can be subtracted from the image, and newly-unobscured Airy disks can be detected using WTMM segmentation.

An out-of-focus particle at any reconstructed depth will exhibit scattering [12]. As such, this method can be used to detect tracks in reconstructed planes without modification. Slight

modifications, such as relaxing constraints on F_c , can be used to detect particles that exhibit non-spherical scattering.

As mentioned in the introduction, the WTMM segmentation method had already been used in a variety of sciences, to identify and segment objects or regions of interest from a noisy background, using a variety of imaging modalities such as fluorescence microscopy, ultra-violet telescopes, magnetogram data, atomic force microscopy, and x-ray imaging (mammography). In this paper, we presented preliminary evidence for the WTMM segmentation method to help make contributions in holography and quantitative phase imaging, by tracking bacteria in different settings.

The value of this method is that it provides a way to identify tracks suggestive of living bacteria with minimal computational burden. The file size of a raw hologram time series is over 100-fold less than a full reconstructed phase stack with a 1.5 mm deep sample chamber. Reconstruction of an ~1100 image time series at 80 depths (corresponding to the 80 scales used in this analysis) alone takes 8 hours, and particles must then be detected in each of these reconstructions. Such considerations are crucial for remote applications, particularly space flight, where total data transmission volume may be restricted and onboard computing power is limited. The Science Definition Team (SDT) report for the proposed Europa Lander mission recommended a microscope with sub-micrometer spatial resolution for detection of possible microorganisms. However, the data budget for the entire mission is 4 gigabits. Methods for collecting pre-compressed data, and on-board pre-processing to determine which data are relayed to Earth, are critical to avoid wasted bandwidth. A combination of holographic microscopy and raw hologram tracking provides a way to obtain a single-bit answer—“Yes” tracks or “No” tracks—that can help determine whether or not a remote instrument has found something of interest.

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Highlights

- A method is proposed to track bacteria from unreconstructed, off-axis holograms.
- The approach is based on the Wavelet-Transform Modulus Maxima segmentation method.
- The method can discriminate between chambers with swimming vs. dead bacteria.
- Further validation could lead to usage for the Europa Lander mission.