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SOFTWARE

HoloBio: A holographic microscopy tool for quantitative biological analysis

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Data availability statement: The HoloBio software is openly available at: https://github.com/SOPHIA-Research-Lab/HoloBio The datasets supporting the findings of this study, including representative holograms and biological sample data, are available at: https://sophia-research-lab.github.io/HoloBio/.

Abstract

Holographic imaging in microscopy enables label-free quantitative information of biological specimens and has found applications across a wide range of biomedical studies, from cell morphology to particle dynamics; yet its widespread adoption is often limited by the lack of accessible and standardized analysis software. We present *HoloBio*, an open-source, Python-based graphical user interface developed to address this issue. This software offers two primary operational modes: a *Real-Time* mode that enables live processing of holograms at video frame rates, and an *Offline* mode designed for post-processing previously recorded holograms. *HoloBio* is compatible with holograms recorded using both lens-based and lensless systems, supporting off-axis architectures in telecentric and non-telecentric configurations, as well as slightly off-axis and in-line optical setups. The software incorporates tools for cell tracking, phase profiling, thickness estimation, and morphological analysis, including cell counting and object area quantification. *HoloBio* is designed to be accessible for users without coding expertise, offering a reproducible, high-throughput environment tailored for researchers in biology, biophotonics, and biomedical imaging.

Introduction

Holographic imaging in microscopy has become a key label-free, video-rate quantitative imaging technique for studying biological specimens because holograms encode both the amplitude (influenced by absorption, attenuation, and scattering) and the quantitative phase (primarily determined by optical path length variations associated with refractive index and thickness) of the optical field scattered by the sample [1]. This capability enables rich, non-invasive characterization of biological samples such as long-term live-cell monitoring and growth assessment [2], quantitative analysis of cell dry mass and thickness changes [3], and automated characterization of morphology and dynamics, including motility and migration trajectories [4]. These imaging

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techniques can also support volumetric inspection and 3D tracking of motile cells and microorganisms, both in lens-based Digital Holographic Microscopy (DHM) and in lensless or in-line configurations (DLHM/DIHM) [5]. The comprehensive quantitative information encoded in DHM and DLHM, spanning optical path length, refractive index, thickness, and morphology, has culminated in a growing body of translational applications, enabling objective, label-free assessment of pathological changes in unstained specimens [6].

This versatility has driven the development of a broad ecosystem of reconstruction and analysis tools across multiple programming environments [7–13]. In the open-source domain, several libraries provide end-to-end holographic processing capabilities, including HoloPy, which supports hologram reconstruction and light-scattering workflows in Python [9], and pyDHM, which implements phase-shifting and phase-compensation pipelines for multiple DHM configurations [8]. At the numerical propagation level, reusable computational backends form the foundation of many DHM and DLHM reconstruction pipelines. For example, CWO++ provides CPU and GPU-accelerated diffraction and propagation routines [12], whereas JDiffraction offers Fresnel and angular-spectrum propagation methods [10]. Beyond core propagation and reconstruction, additional projects address higher-level holography workflows. OpenHolo supports hologram generation, reconstruction, and signal processing [13]. DHM capabilities have also been integrated into widely adopted biomedical imaging platforms through ImageJ-based toolsets, including HoloJ and other DHM-focused plugins for simulation, reconstruction, and analysis workflows [11,14]. Similar ImageJ-based approaches have been extended to lensless and in-line holographic configurations [15]. Complementing these open-source efforts, commercial platforms, including Holo4D and other proprietary DHM software suites [16,17], provide fully integrated reconstruction and analysis environments, underscoring the maturity and broad adoption of digital holographic microscopy across both research and translational settings. Additionally, tools such as pylorenzmie provide GUI-based hologram analysis frameworks tailored to specific applications, such as colloidal particle characterization, further illustrating the diversity of available approaches within the field [18].

However, despite their technical contributions, existing tools present important limitations that hinder broader adoption. Many libraries require advanced programming expertise and a strong background in Optics, thereby limiting their accessibility to researchers in biological and clinical environments. Plugin-based solutions, while widely popular in the biomedical imaging community, tend to be task-specific and often lack cohesive integration across hologram acquisition, reconstruction, and downstream biological quantification. Commercial software platforms offer robust and polished functionalities, but their closed and proprietary nature limits accessibility and widespread use in academic research. As a result, a significant usability gap still remains for biomedical researchers who could otherwise benefit from the versatility of DHM and DLHM but are impeded by the complexity and fragmentation of existing software ecosystems. The lack of a unified, biology-oriented graphical user interface continues to constrain the broader adoption of DHM and DLHM in translational

applications such as cell biology and histopathology, where extracting quantitative phase and morphological information from label-free samples holds significant scientific and clinical relevance.

To address these challenges, we present *HoloBio*, a free and open-source Python-based graphical interface software specifically designed for biological applications using DHM and DLHM. *HoloBio* supports a broad range of experimental configurations and integrates multiple reconstruction strategies tailored to different optical geometries, including in-line, slightly off-axis, and off-axis holography. The software is compatible with both real-time (video-rate) data acquisition and the processing of previously recorded holograms. Beyond hologram reconstruction, *HoloBio* provides a suite of biology-oriented tools aligned with standard microscopy workflows, including micrometric image scaling, digital refocusing via numerical propagation, quantitative phase mapping, thickness estimation, phase profiling and particle tracking. In addition, the platform also features biological analysis utilities such as particle and cell counting, morphology assessment, and speckle noise reduction, enabling robust and reproducible interpretation of label-free imaging data.

Design and implementation

Overview of DHM and DLHM modalities

Digital Holographic Microscopy (DHM) and Digital Lensless Holographic Microscopy (DLHM) are two widely used configurations for quantitative phase imaging (QPI), which primarily differing in their optical implementation and image-formation principles. DHM relies on a microscope-based architecture that incorporates imaging optics and a reference beam, enabling controlled magnification, separation of diffraction orders, and stable phase reconstruction under well-defined conditions. In contrast, DLHM removes imaging lenses and records diffraction patterns directly on the sensor, resulting in more compact, simpler systems but with a greater dependence on numerical reconstruction and accurate system parameter estimation.

As shown in Fig 1, the Basic DHM setup corresponds to an interferometric microscope configuration with imaging optics and a reference beam, whereas the Basic DLHM setup is a lensless arrangement in which diffraction patterns are recorded directly on the sensor. These differences in optical configuration determine the subsequent numerical

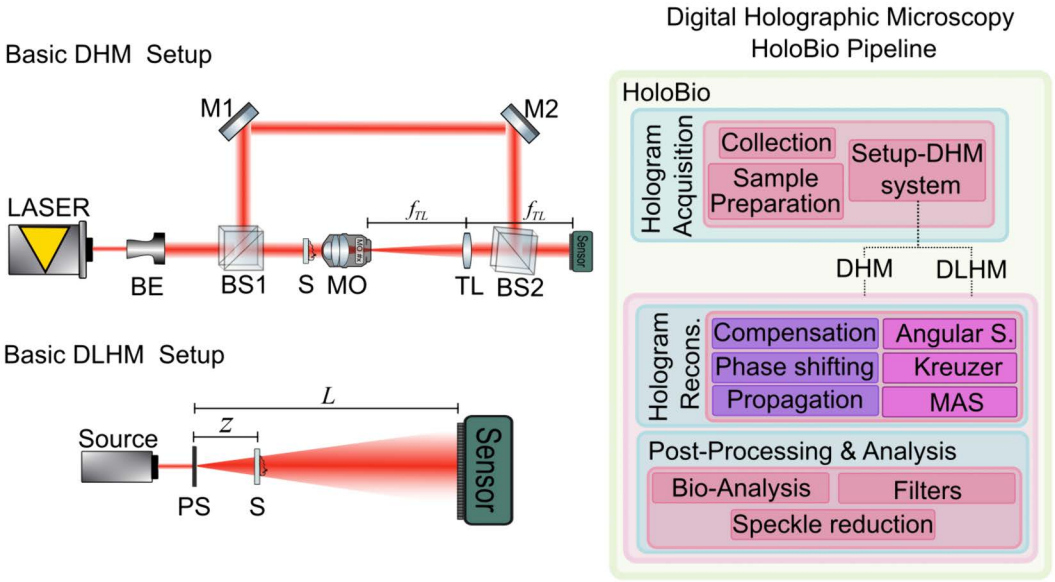

Fig 1. Overview of DHM and DLHM configurations and HoloBio processing pipeline. Simplified optical setups for DHM and DLHM, together with the HoloBio workflow, including hologram acquisition, numerical reconstruction, and post-processing stages.

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reconstruction and processing workflows. The schematic representations highlight the fundamental principles of each modality. Despite these differences, both DHM and DLHM follow a common conceptual workflow, which can be divided into three main stages as Fig 1 shows: (i) hologram acquisition, (ii) numerical reconstruction, and (iii) post-processing and analysis. The acquisition stage involves the preparation and recording of holograms using the corresponding experimental setup, which remains external to the HoloBio platform. This acquisition stage is followed by the reconstruction stage, where computational methods such as phase-shifting, phase compensation, or numerical propagation are applied to reconstruct the complex optical field. Finally, post-processing tools are used for tasks such as filtering, speckle reduction, and quantitative bio-analysis.

In this context, HoloBio integrates the reconstruction and post-processing stages into a unified computational framework, providing tailored pipelines for both DHM and DLHM modalities. This modular organization allows users to process holographic data under different experimental conditions while maintaining a consistent and flexible analysis environment.

HoloBio is structured around two main operational modes: (1) *Real-Time* Hologram Processing and (2) *Offline* Hologram Processing. Each mode is subdivided into two specialized packages corresponding to the holography modalities: Digital Holographic Microscopy (DHM) [1] and Digital Lensless Holographic Microscopy (DLHM) [19]. This modular organization ensures that each functional interface is optimized for a specific optical configuration and processing workflow.

Initialization and mode selection

Upon launch, HoloBio presents a graphical welcome interface that guides users to intuitively select the operational mode and digital holography modality (Fig 2). Depending on the selected modality, the software dynamically loads the corresponding interface, optimized for either live acquisition or reconstruction of previously recorded holograms.

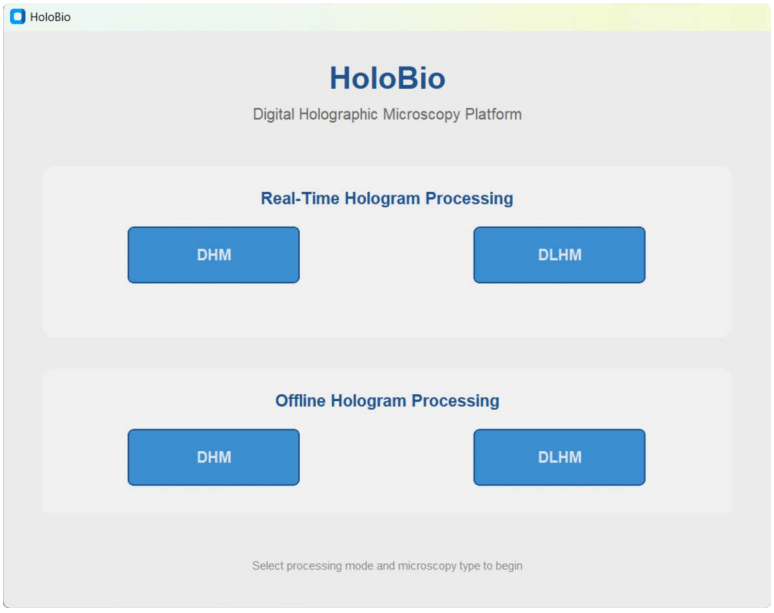

Fig 2. Startup interface of HoloBio. The welcome screen allows users to select between Real-Time or Offline processing modes and choose the desired microscopy modality (DHM or DLHM).

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Real-Time Hologram processing

This mode allows users to live-acquire and reconstruct holograms using a digital camera connected to the system. For both DHM and DLHM imaging modalities, the software provides real-time video-rate visualizations of amplitude and phase-compensated reconstructed images. In this package, users can also import previously recorded hologram videos for reconstruction, as well as amplitude or phase images for particle tracking. Additionally, individual frames can be processed for quantitative phase analysis, speckle noise reduction and conventional image enhancement.

***Real-Time* DHM package:** This package is designed for DHM systems operating in off-axis configuration under telecentric regime [20]. It provides a live interface for real-time visualization of raw holograms alongside their corresponding reconstructed amplitude and phase images. These reconstructed images are computed frame-by-frame using the semi-heuristic phase compensation (SHPC) method [21]. Additional features include real-time display of the hologram Fourier Spectrum with interactive zoom, allowing inspection of specific regions in spatial or frequency domain. This capability is particularly useful for system alignment and fine-tuning of telecentric conditions. Both raw holograms and reconstructed amplitude or phase images can be recorded as video sequences. Additionally, previously recorded hologram videos can be imported for real-time reconstruction, supporting both live and retrospective analysis.

The *Real-Time* DHM package is designed to support an intuitive workflow for hologram acquisition, reconstruction, and analysis (Fig 3). Users begin by defining the physical parameters required for reconstruction, such as wavelength and pixel pitch, through the parameter panel. The interface then provides real-time visualization tools, including Fourier transformation, in which spatial filtering can be configured and inspected interactively. Once configured, users can execute real-time reconstruction, perform continuous acquisition, and record sequences of holograms or reconstructed amplitude and phase images. Additional functionalities, such as particle tracking, enables analysis of previously recorded holograms, extracting frame-by-frame particle positions. The visualization panel integrates these processes by displaying the hologram or its Fourier spectrum alongside the reconstructed amplitude or phase, with interactive zoom and view adjustment capabilities, facilitating real-time interpretation and system alignment.

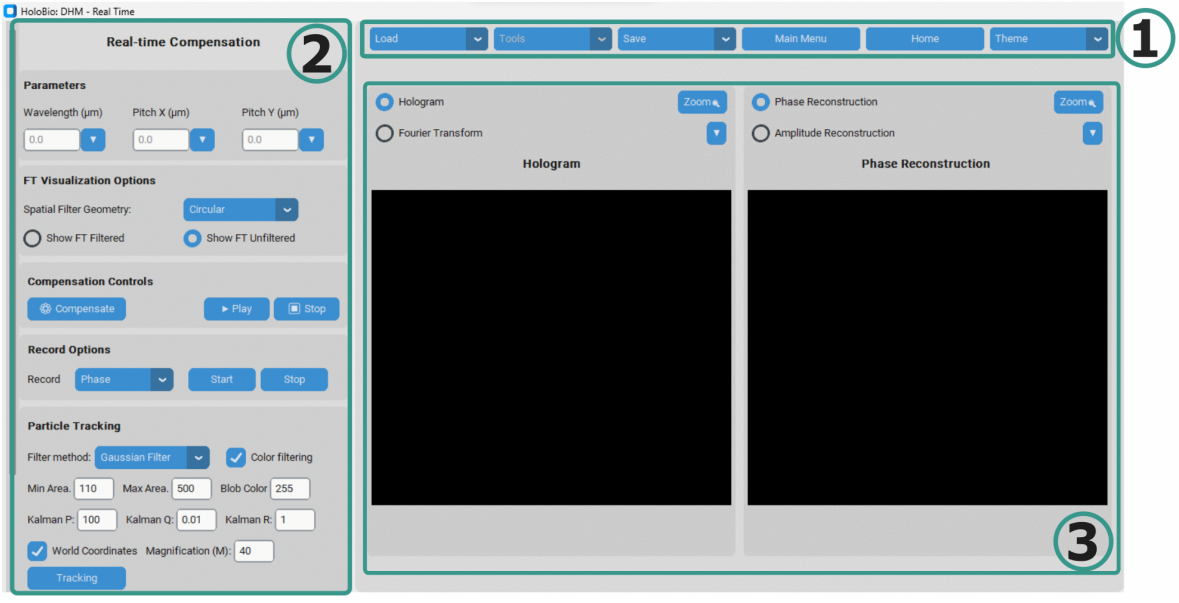

Fig 3. Interface for the Real-Time DHM package in HoloBio. The interface is organized into three components: (1) the control panel with core functionalities, (2) the real-time processing panel. (3) The visualization panel for hologram and reconstruction display.

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***Real-Time DLMH* package:** This package is intended for DLHM systems employing either spherical (Point-source DLHM) [19] or planar (On-Chip Lensless systems) [22] illumination. It supports real-time visualization of holograms and live reconstruction, providing amplitude and phase outputs. Users can select among three computationally-efficient reconstruction algorithms: the Angular Spectrum approach for plane-wave propagation [23], a discrete version of the Kirchhoff–Helmholtz diffraction integral accounting for spherical wavefronts (KHDI) [15], and a modified Angular Spectrum approach for spherical illumination (MAS) [24]. A distinctive feature of this package is the interactive control of the propagation distance, enabling real-time adjustment to achieve optimal focus.

Offline Hologram Processing

The *Offline* mode is dedicated to the numerical reconstruction and analysis of previously recorded holograms. Upon selection, users choose between DHM and DLHM modalities, after which the software launches a specialized interface tailored to the corresponding optical configuration.

***Offline* DHM package:** This package offers three reconstruction workflows: *Phase-Shifting*, *Phase Compensation*, and *Numerical Propagation*. The *Phase-shifting* workflow supports six algorithms to reconstruct in-line or slightly off-axis holograms, including the 5-Frames [25], 4-Frames [26], 3-Frames [25], and Quadrature [27] methods, as well as two blind approaches for unknown phase shifts: Blind 3 Raw Frames [28] and Blind 2 Raw Frames [29]. The *Phase Compensation* workflow provides four methods for off-axis holograms: Semi-Heuristic Phase Compensation (SHPC) [21], Tu-DHM [30], Non-Telecentric reconstruction [31], and Vortex-Legendre fitting [32]. Finally, the *Numerical Propagation* workflow enables reconstruction at arbitrary propagation distances for experimental or simulated wavefields using either the Angular spectrum method or the paraxial Fresnel Transform [23].

Selecting this package opens the corresponding interface shown in Fig 4, which is designed to guide the user through the hologram reconstruction workflows described previously. The interface comprises three main components. At the top of the interface, the main control panel (1) provides access to core software functionalities. This includes buttons

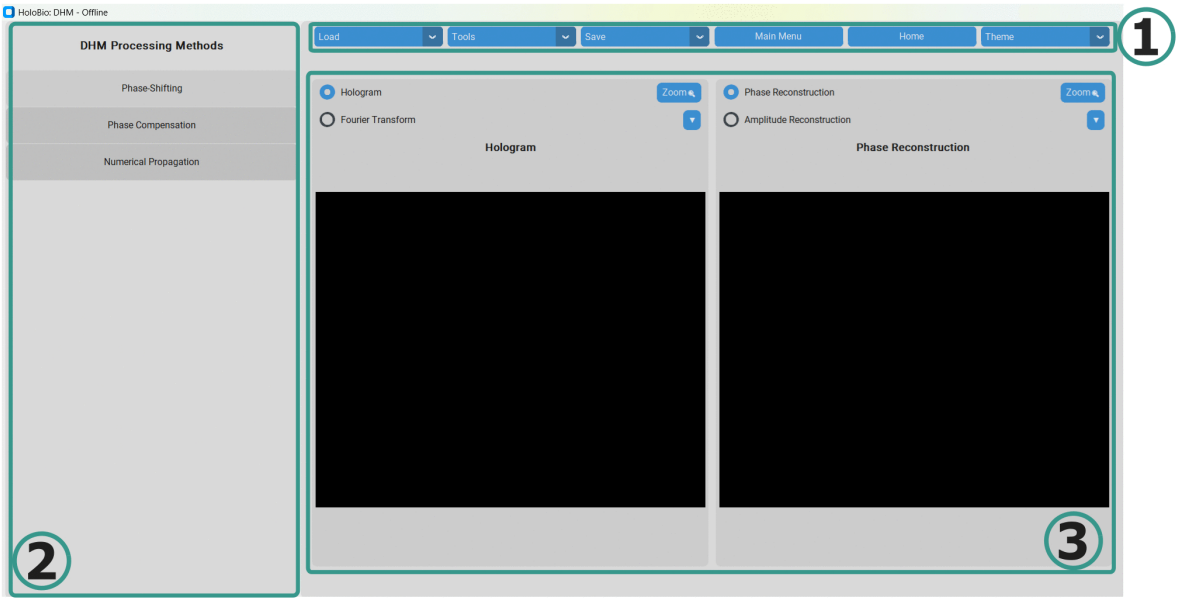

Fig 4. Offline DHM package interface in HoloBio. The interface is organized into three components: (1) the control panel with core functionalities, (2) the method selection panel, and (3) the visualization panel for hologram and reconstruction display.

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for loading and saving data, accessing additional tool panels (*Tools*), navigating to the main menu or home screen, and switching between interface themes. The left panel (2) provides access to the available DHM processing methods: *Phase Shifting*, *Phase Compensation*, and *Numerical Propagation*. Depending on the selection, the control panel dynamically adapts to display relevant parameters and tools for the chosen method. The visualization panel (3) serves as the main visualization area, where users can display the original hologram, its Fourier transform, and the corresponding amplitude or phase reconstruction.

***Offline* DLHM package:** This package provides the same three reconstruction algorithms available in *Real-time* mode: Angular Spectrum, KHDI, and MAS. The interface enables interactive adjustment of the source-to-camera (*L*) and source-to-sample (*z*) distances, thereby facilitating identification of the correct focal plane when the reconstruction distance is unknown a priori. Additionally, users may load a sample-free reference hologram for subtraction from the sample hologram, which significantly reduces background noise and twin-image artifacts in amplitude reconstructions.

Selecting this package opens the interface shown in Fig 5. The interface is also organized into three components. The top control panel (1) contains the primary navigation and management tools, including options to load and save data, access tool menus, return to the main menu or home screen, and change the interface theme. The parameter panel (2) allows the user to configure DLHM-specific reconstruction settings, such as wavelength, pixel pitch in X and Y, magnification, and *z* and *L* distances. Additional controls are available to select the reconstruction algorithm (Angular Spectrum, KHDI, or MAS), define reconstruction limits, and apply hologram reconstruction. The visualization panel (3) displays the selected outputs, enabling users to view the original hologram or its Fourier transform, alongside either reconstructed amplitude or phase images.

Shared post-processing and analysis tools

For each operational mode, *HoloBio* provides a set of tools.

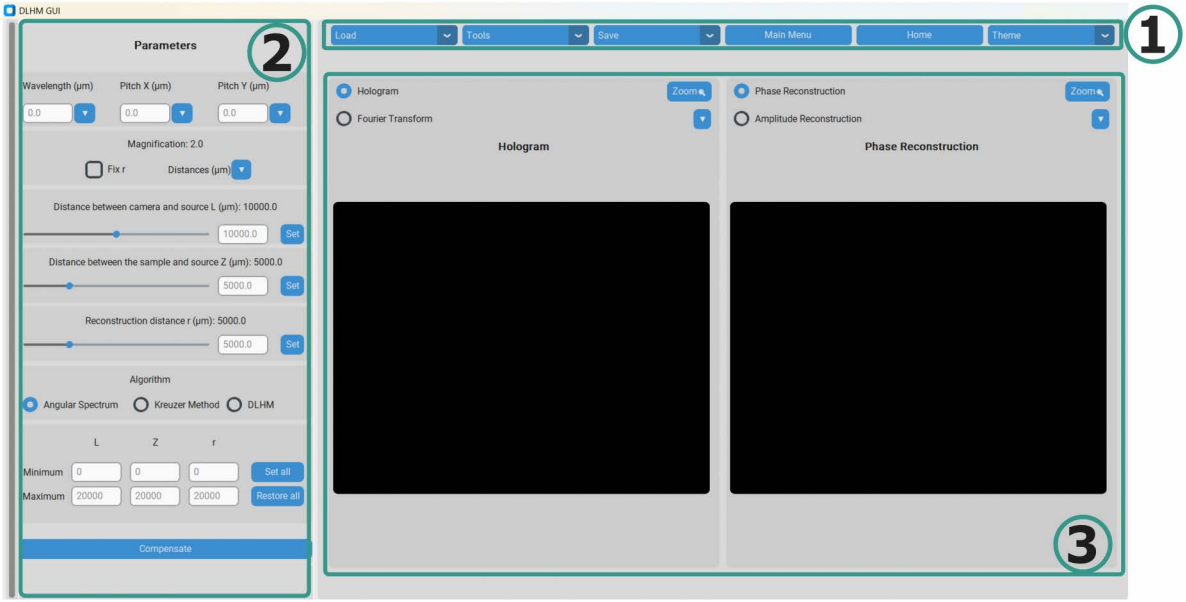

Fig 5. Interface for the Offline DLHM package in HoloBio. The interface is organized into three components: (1) the control panel with core functionalities, (2) the parameter and algorithm selection panel, where users can set physical parameters (wavelength, pixel pitch, z and L distances) and choose among available reconstruction methods, and (3) the visualization panel for hologram and reconstruction display.

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- **Bio-Analysis Toolkit**: Designed for biological/biomedical quantification, it allows users to measure phase shifts, extract phase profiles, perform length and thickness measurements, count particles/cells, and estimate sample areas, enabling comprehensive morphological and dimensional analysis.
- **Filters Toolkit**: Enhances image contrast through a variety of spatial filters and colormaps, improving the visualization and interpretation of both amplitude and phase reconstructions.
- **Speckle Toolkit**: Provides tools for the quantification and reduction of speckle noise in selected regions of interest, whether from amplitude, phase, or raw hologram images. Available denoising methods include hybrid median filter [33], mean filter [34], median filter [34], Gaussian filter [34], and SPP filter [35]. The toolkit also incorporates comparative visualization features (before/after filtering), speckle reduction plots, and profile extraction to evaluate filtering performance.

To provide a unified view of the platform, Fig 6 summarizes the overall architecture of HoloBio, integrating both DHM and DLHM modalities within real-time and offline processing modes. The diagram highlights how acquisition, reconstruction, and visualization modules are organized within each workflow, together with the set of shared tools available for post-processing and quantitative analysis.

Results

Quantitative biological imaging with *HoloBio*

This section presents biological imaging applications of *HoloBio*. Each application highlights specific functionalities of the software, demonstrating the utilities of each module and submodule and how the platform supports key tasks such as quantitative phase analysis, cell counting, morphological profiling, and object tracking.

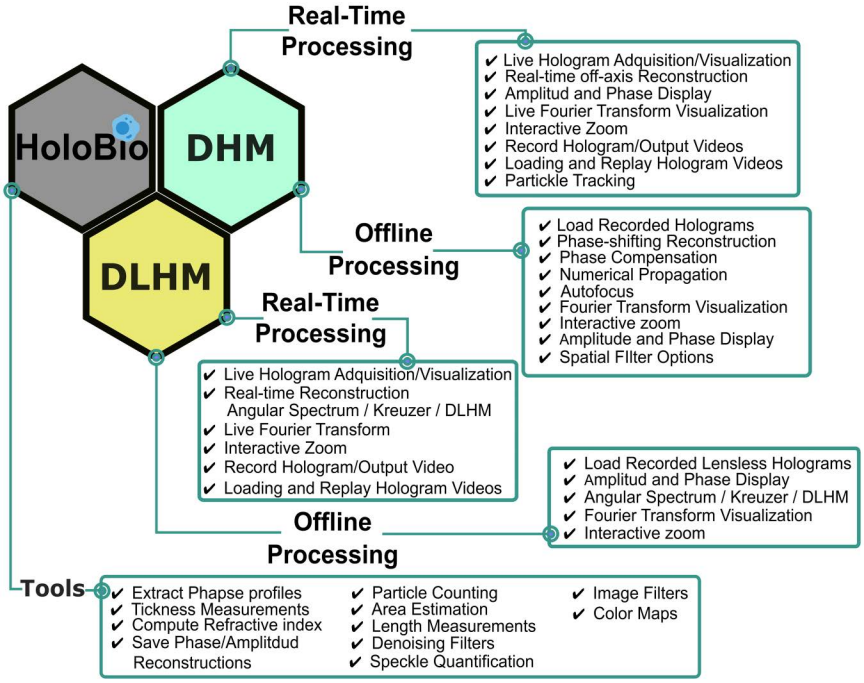

Fig 6. Architecture of HoloBio. The diagram illustrates the integration of DHM and DLHM within the HoloBio platform, organized into two main operational modes: real-time and offline processing. Each mode provides dedicated modules for hologram acquisition, numerical reconstruction, visualization, and quantitative analysis through integrated toolsets.

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Imaging of defocused Red Blood Cell samples

To evaluate *HoloBio* on experimentally defocused off-axis DHM data, we imaged Red Blood Cells (RBC) located at different axial positions from the working distance of the microscope objective (MO) lens. This experiment comprises two steps: (1) numerical reconstruction and phase compensation of the recorded holograms, and (2) autofocusing of the recovered complex wavefield. The RBCs were obtained from Carolina Biological Supply Company (item # C25222) and imaged using a telecentric off-axis DHM system based on a common-path interferometer [36]. Illumination was provided by a low-power 532-nm laser diode module (CPS532, Thorlabs). The imaging system includes a 40×/0.75 NA infinity-corrected Nikon MO lens and a 200-mm tube lens. Holograms were recorded using a digital camera with a resolution of 5472×3648 pixels and a 2.4-μm pixel pitch. Controlled defocus was introduced by axially translating the sample with a micrometer translation stage.

Using the Offline DHM package, phase compensation follows a structured workflow that enables the retrieval of amplitude and phase information from experimentally defocused holograms (Fig 7). The process begins by selecting an appropriate compensation method, chosen according to the optical configuration of the system. In this demonstration, the Vortex–Legendre fitting method was employed. The reconstruction parameters, including the illumination wavelength and pixel pitch, are defined based on the experimental setup. Subsequently, a spatial filtering stage is applied in the Fourier domain to isolate the +1 diffraction order, ensuring proper separation of the object information. Here, an automatic circular filter was selected. These configuration steps are illustrated in the interface panel shown in Fig 7A. Once the parameters are defined, the compensation process is executed, leading to the retrieval of the complex wavefield. A visual confirmation of the selected filtering region is provided through the filtered Fourier transform (Fig 7B). The progression of the reconstruction is shown in Fig 7C–E, where the original out-of-focus hologram (C), the initial phase reconstruction (D), and the final numerically refocused phase image (E) illustrate the effectiveness of the compensation and refocusing process.

In section (iv), users configure the **Propagation Options** subpanel to obtain in-focus images, either in amplitude or in phase. The panel offers three modes: Fixed Distance, Z-scan Sweep, and Auto Focus. In this example, the Auto Focus mode is selected. In this mode, the user defines a minimum and maximum axial propagation range over which the software performs numerical propagation to determine the optimal focus. The optimal focus distance is assessed using one of the two built-in sharpness metrics: Normalized Variance or Tenengrad metric [37]. The default metric is the Normalized Variance. Additionally, the user must input the **lateral magnification** of the optical system, in this case 40×, to scale the focus distance correctly to the object space. Once the user clicks the Apply button, a sequence of guided steps is triggered: Once the user clicks the Apply button, a sequence of guided steps is triggered: First, a window appears prompting the user to select a Region of Interest (ROI) from the reconstructed image (Fig 8A). The sharpness metric will be computed exclusively within this selected region. After the ROI is selected, a second pop-up window appears, displaying the Auto-Focus in Progress indicator (Fig 8B), while the algorithm evaluates different propagation distances within the specified range. If the “Show Curve” option is enabled, the software displays the focus curve, which plots the sharpness metric as a function of propagation distance (Fig 8C). In this example, the minimum of the curve occurs at 13.47 μm, which is identified as the optimal propagation distance [38].

Once the hologram is properly compensated and focused, the *Bio-Analysis toolkit* provides a set of tools designed for the quantitative assessment of reconstructed amplitude and phase images. As shown in Fig 9A, the panel is divided into three blocks: (i) *Dimensions*, (ii) *QPI Measurements*, and (iii) *Microstructure Metrics*.

Dimensions: This block enables users to quantify spatial dimensions within the reconstructed image. First, the user selects the image type to analyze using the radio buttons: *Hologram*, *Amplitude*, or *Phase*. This choice determines the image on which the dimensional measurement is performed. Next, two key physical parameters must be entered: the *Pixel Size* (in micrometers), which corresponds to the pixel pitch of the digital camera used to acquire the hologram, and the *Lateral Magnification*, which refers to the effective optical magnification applied in the imaging system. In this specific example, a pixel size of 2.4 μm and a lateral magnification of 40× were used. After clicking the *Apply* button, a pop-up window displays the selected image with an overlaid scale bar, as shown in Fig 9B. To perform a measurement,

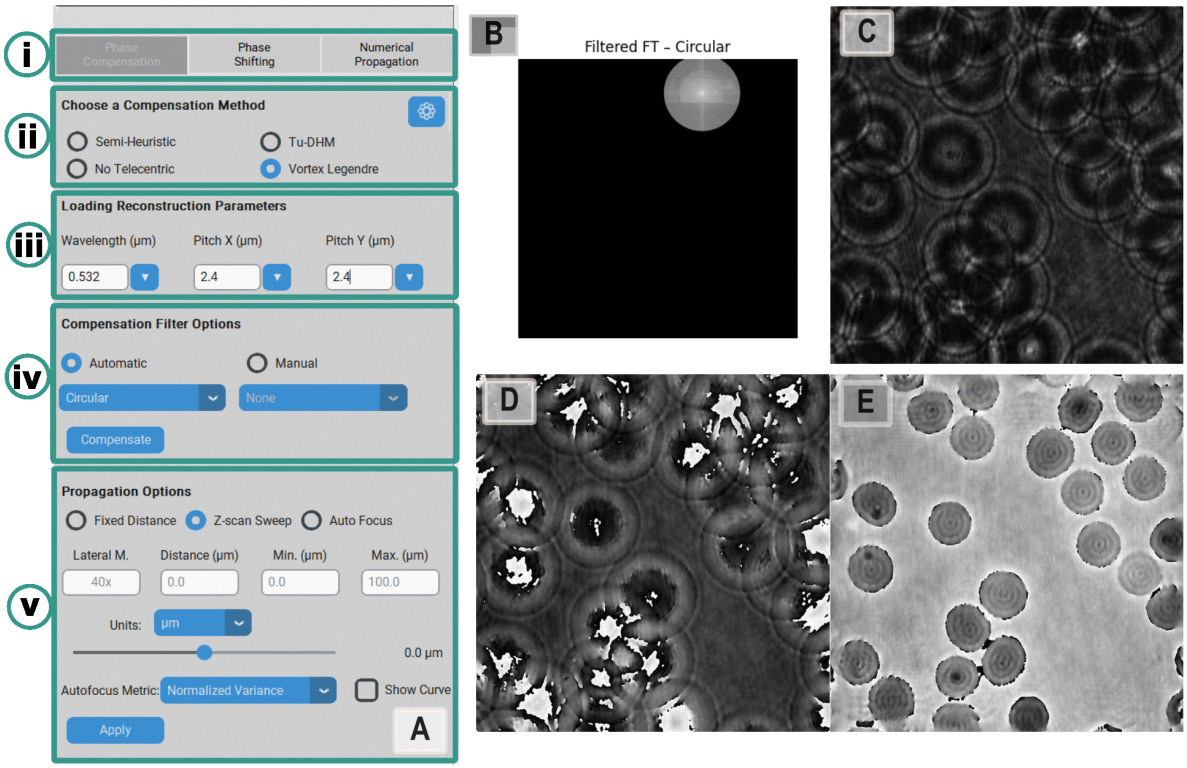

Fig 7. Workflow for phase compensation and numerical refocusing in HoloBio using the Offline DHM Processing package. (A) Interface panel structured into five functional blocks: (i) phase compensation method, (ii) reconstruction parameters, (iii) compensation filter options, and (iv–v) propagation settings. (B) Pop-up window showing the filtered Fourier transform with the isolated +1 diffraction order. (C) Original out-of-focus hologram. (D) Initial phase reconstruction (still out of focus). Finally, (E) numerically refocused phase image after compensation.

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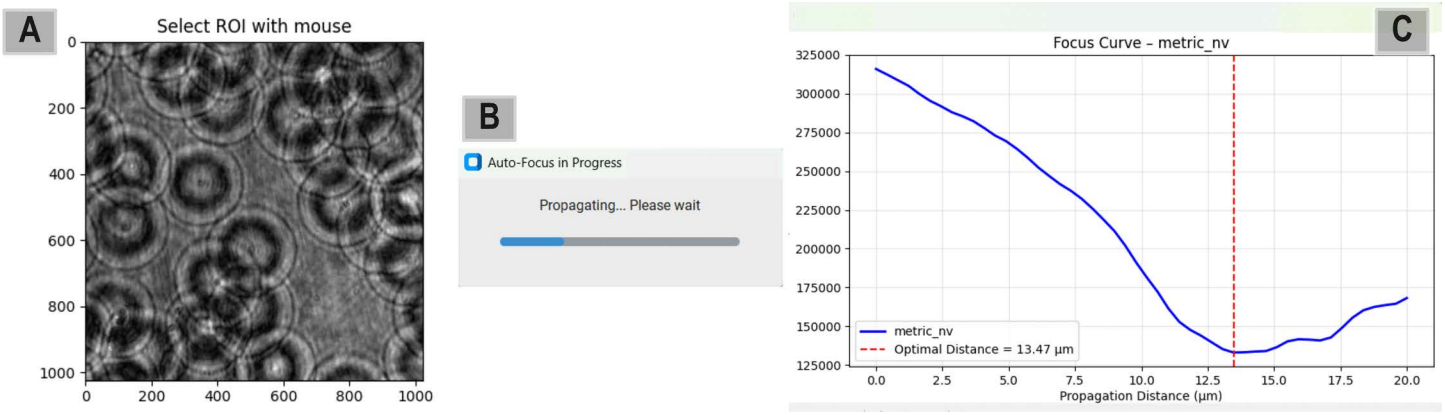

Fig 8. Autofocus workflow in HoloBio. (A) A window to select a region of interest (ROI) from the reconstructed image. (B) Auto-Focus progress indicator during propagation. (C) Focus curve showing the propagation distance versus focus metric, with the optimal focal plane indicated.

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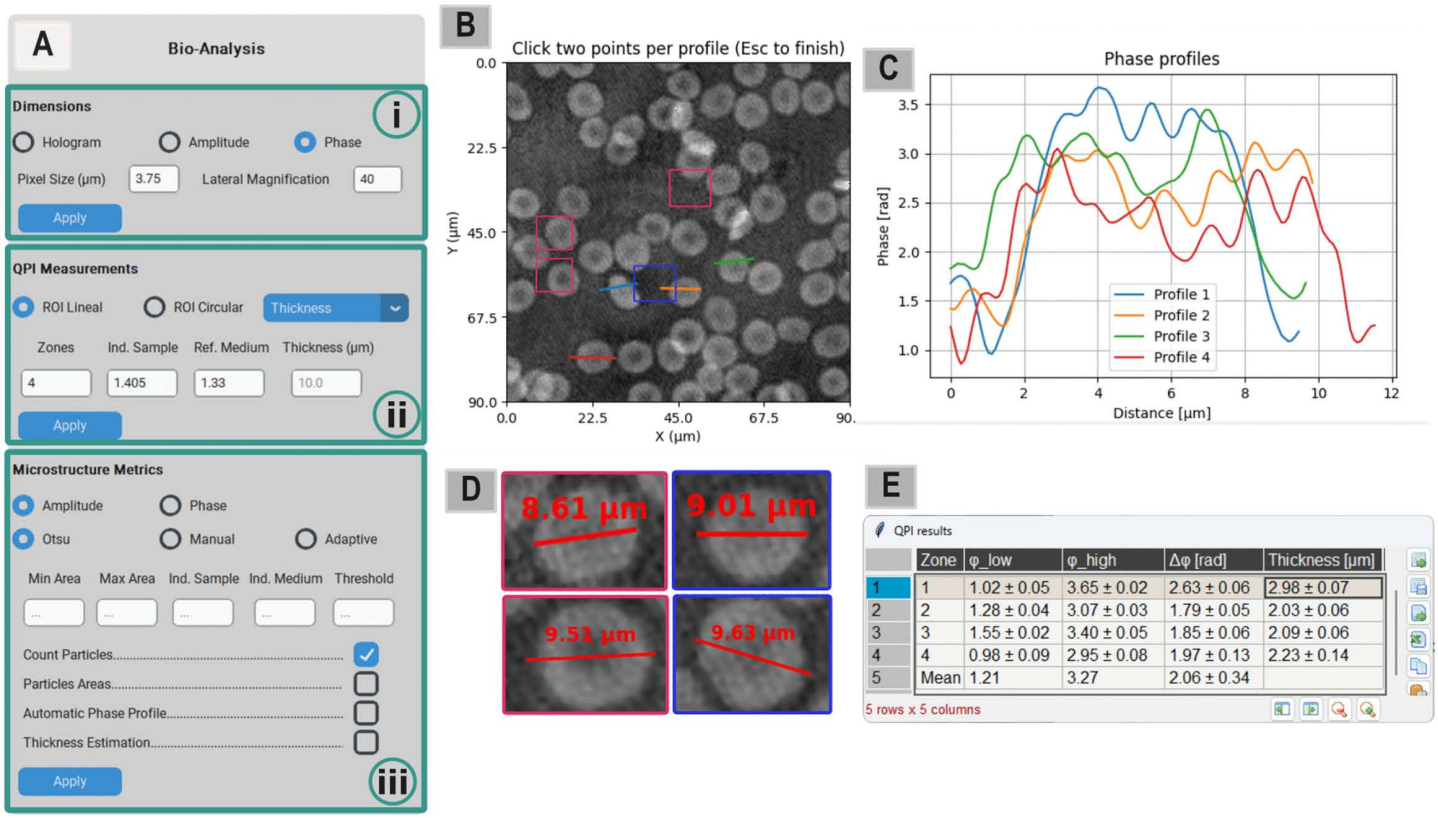

Fig 9. Bio-Analysis workflow in HoloBio, showing an example application for dimensional analysis and QPI measurements. (A) Main Bio-Analysis panel with options for dimensions, QPI measurements, and microstructure metrics. (B) Phase image of the sample with dimensional measurements indicated. (C) Window for selecting regions of interest (ROI) directly on the phase image. (D) Phase profiles extracted from the selected ROIs. (E) Summary table of QPI results with corresponding thickness values and example dimensional annotations of the sample.

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the user clicks on two points of interest in the image; a straight line is drawn between the selected positions, and the corresponding distance in micrometers is displayed on screen. In the current example, two RBCs were measured, with zoomed-in regions shown at the bottom for clarity. The results indicate diameters of approximately 8.35 µm and 6.98 µm, respectively. Additionally, the field of view is expressed in micrometers (µm), providing a more intuitive spatial context for interpreting the measurements.

QPI Measurements. This block is designed to perform Quantitative Phase Imaging (QPI) measurements on previously reconstructed phase images. All QPI-related configurations are performed within section (ii) of the Bio-Analysis panel (Fig 9A), which includes the selection of the ROI type (*ROI Lineal* or *ROI Circular*), the measurement mode (*Thickness* or *Index*), and the corresponding physical parameters. In this example, the analysis focuses on thickness estimation of RBCs. The parameters were set to: *Zones* = 4, which defines the number of phase profiles (i.e., the number of analyzed cells); *Ind. Sample* = 1.405, corresponding to the refractive index of the sample (erythrocytes); and *Ref. Medium* = 1.33, representing the refractive index of the surrounding medium. After clicking the *Apply* button, the user defines the regions of interest by selecting two points per profile directly on the reconstructed phase image (Fig 9B), where each line corresponds to a different erythrocyte. Once the ROIs are defined, the software computes the phase profile for each selected region. The resulting profiles are shown in Fig 9C, where each curve corresponds to a different cell (Profile 1–4). The estimated thickness values derived from these profiles are: 2.98 ± 0.07 µm (Profile 1, blue),

2.03 ± 0.06 μm (Profile 2, yellow), 2.09 ± 0.06 μm (Profile 3, green), and 2.23 ± 0.14 μm (Profile 4, red). These results are summarized in the table shown in Fig 9E, which reports the minimum (φ_low), maximum (φ_high), phase difference (Δφ), and thickness for each zone, along with the mean and standard deviation. Red blood cells exhibit a characteristic biconcave morphology, with typical thickness values in the range of 1.7–2.2 μm for *discocytic* cells [39,40]. The thickness values obtained in this work fall within this range, indicating that the reconstructed phase maps capture meaningful morphological features. These results highlight the capability of the proposed platform to provide quantitative thickness information, beyond conventional 2D intensity-based measurements.

Microstructure Metrics. The Microstructure Metrics block provides tools for morphological analysis of biological samples, including *Count Particles*, *Particle Areas*, and *Thickness Estimation* (Fig 9A-iii). In this case study, the analysis focused on the first two functionalities. Regardless of the selected option, users must choose one of three segmentation strategies: *Otsu*, *Manual*, or *Adaptive* to isolate relevant microstructures. Additionally, the system allows specification of minimum and maximum area thresholds to filter irrelevant features. For the RBCs sample, this module is used to estimate the projected area of individual cells by selecting the *Phase* image, applying the *Otsu* thresholding method for segmentation, and enabling the *Particles Areas* option. Upon clicking the *Apply* button, the software first requests confirmation of the segmentation polarity, then displays the resulting binary mask and the corresponding intensity histogram with the computed threshold overlaid (Fig 10A, B, and D). The detected connected components are subsequently filtered according to the specified area constraints, and the valid RBCs are outlined and labeled in the processed mask (Fig 10C).

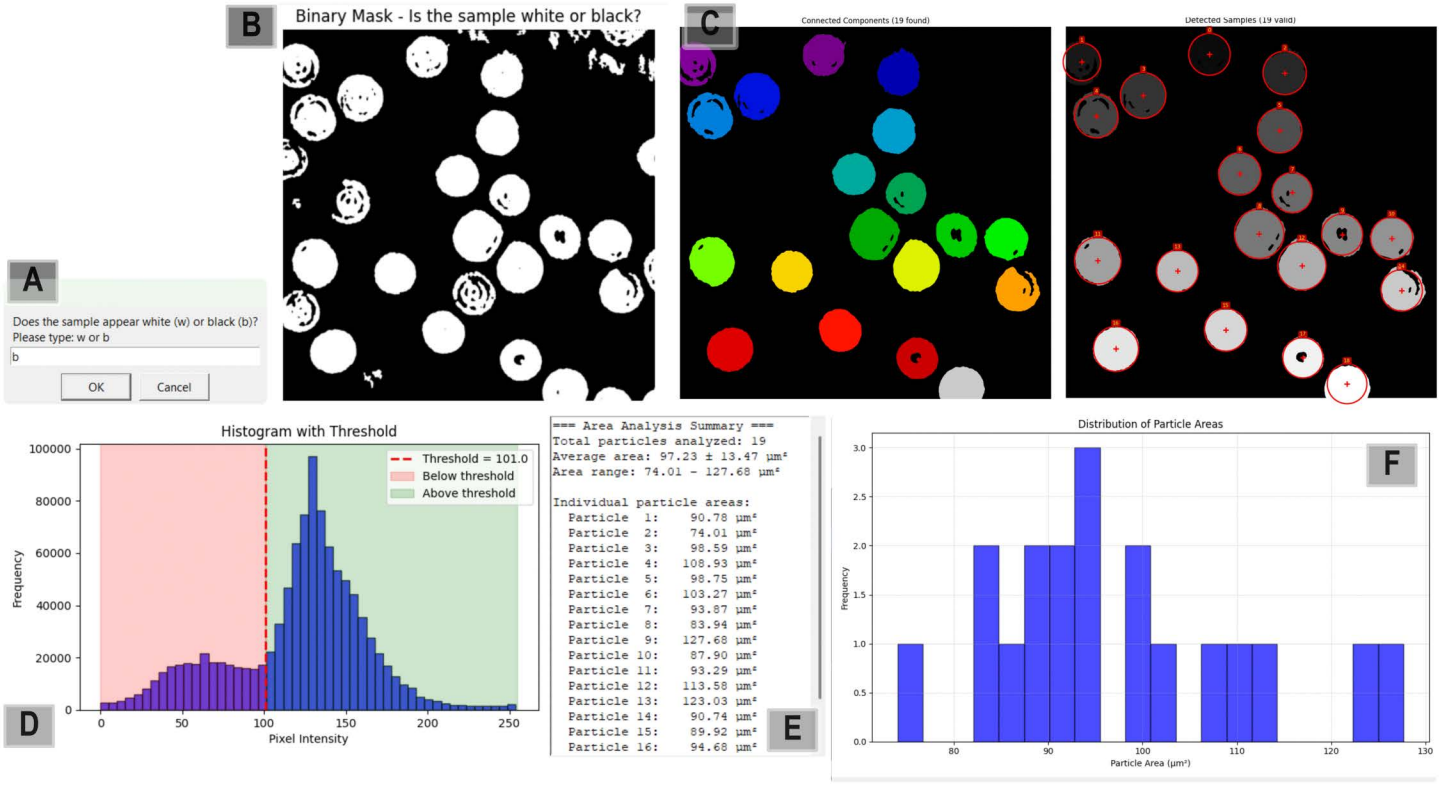

Fig 10. Bio-Analysis workflow in HoloBio, showing an example application for particle area estimation. (A) Confirmation dialog for sample polarity. (B) Binary mask obtained after segmentation. (C) Connected components detected (left) and valid particles after filtering by area constraints (right). (D) Histogram of pixel intensities with the computed threshold. (E) Area Analysis Summary window with numerical results of individual particles. (F) Histogram of particle area distribution.

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The Area Analysis Summary (**E**) provides numerical information for each individual particle, including the total number of particles (19), the average area (97.23 ± 13.47 μm²), and the area range (74.01–127.68 μm²). Additionally, the particle area distribution is shown as a histogram in Fig 10F, illustrating the size variability of the detected RBCs.

Speckle noise reduction of onion epidermis tissue samples

An additional biological use case in which *HoloBio* demonstrates its utility for enhancing reconstructed images is speckle noise reduction. As an illustrative example, a set of three phase-shifted holograms of onion epidermis tissue was acquired in a slightly off-axis configuration [41]. The reconstruction was performed using the *Offline* DHM package with the *Phase-Shifting* option, specifically the *Blind 3 Raw Frames* method. The reconstruction parameters were set to a wavelength of λ = 632.8 nm and a pixel pitch X and Y of 5.2 μm, providing both amplitude and phase reconstructions suitable for quantitative analysis.

Fig 11A presents the Phase-Shifting interface, highlighting (i) the *Offline* DHM menu options, (ii) the panel for selecting the phase-shifting method from the available modes (see Design and Implementation section), (iii) the parameter input panel for reconstruction setting, including wavelength and pixel pitch, and (iv) the Propagation Options panel. Fig 11B, C show one of the recorded phase-shifted holograms and its corresponding Fourier Spectrum, respectively. The blue rectangles in panel (B) display the two additional phase-shifted holograms used in the reconstruction process. The Fourier Spectrum in panel (C) confirms the slightly off-axis configuration since the spectral orders overlap. The reconstructed amplitude and phase images are shown in panels (D) and (E). respectively, after applying the Blind 3 Raw Frames method.

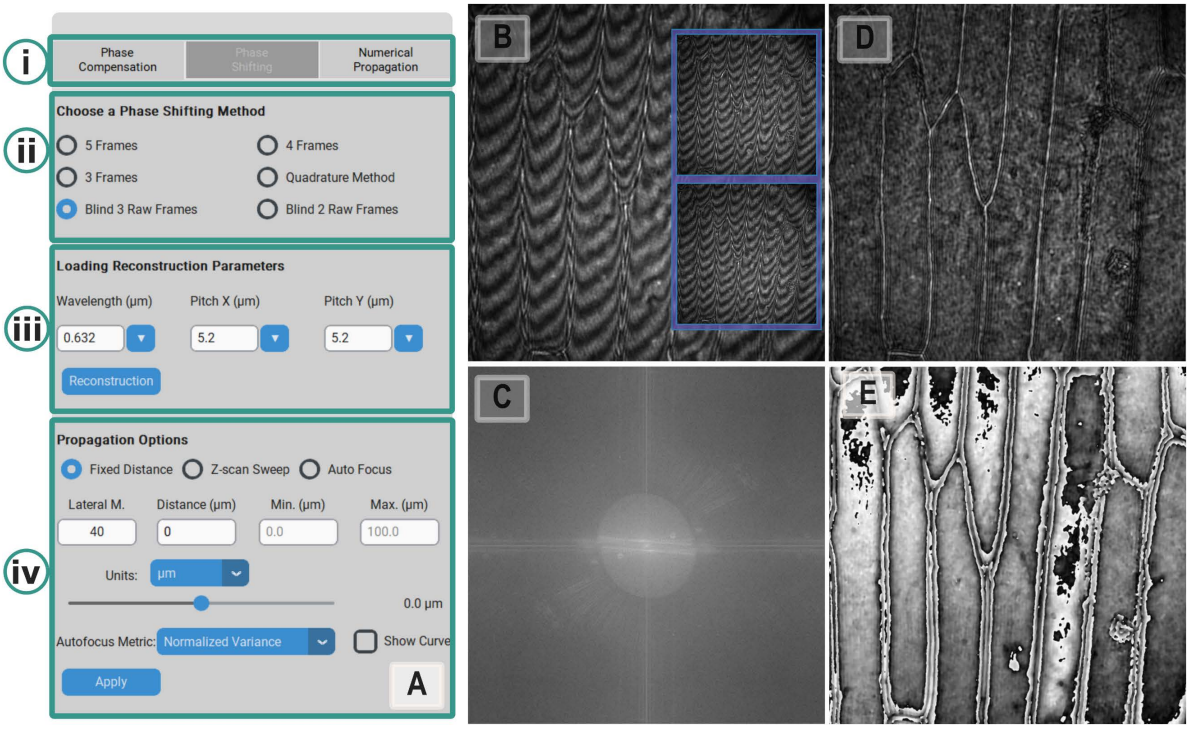

Fig 11. Phase-shifting reconstruction workflow of onion epidermis tissue samples using HoloBio. (A) Phase-Shifting interface showing the main menu options, phase-shifting method selection, reconstruction parameters, and propagation options. (B) Example of one recorded hologram, with blue rectangles illustrating fringe shifts. (C) Fourier transform of the hologram, confirming the slightly off-axis configuration. (D) Amplitude reconstruction obtained using the Blind 3 Raw Frames method. (E) Corresponding phase image reconstruction.

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Speckle analysis is performed using the *Speckle Toolkit* integrated into *HoloBio* (Fig 12A). The workflow begins by selecting the image type (hologram, amplitude, or phase) and defining regions of interest (Zones) for localized analysis. Each selected region can be subdivided into smaller sub-regions, enabling a detailed spatial characterization of speckle behavior. Speckle noise is quantified using the speckle contrast metric, defined as the ratio between the standard deviation and the mean intensity within each sub-region [42]. This approach enables both local and global evaluation of speckle characteristics across the image. Following parameter definition, speckle reduction methods can be applied using configurable filtering strategies. The effectiveness of the filtering process is illustrated through comparative visualizations (Fig 12B), where the original and denoised images are displayed side by side. The yellow highlighted regions in Fig 12B correspond to zoomed areas that emphasize local differences in speckle patterns, facilitating visual assessment of the noise reduction performance.The spatial distribution of speckle is further analyzed through the selected regions and their corresponding subdivisions (Fig 12C), where each zone is partitioned into smaller elements to enable localized quantification. This subdivision allows the evaluation of spatial variations in speckle contrast across the image. Quantitative assessment is provided by comparing intensity profiles before and after filtering (Fig 12D), showing a clear reduction in fluctuations and improved uniformity in the denoised signal. Additionally, the computed speckle contrast values for each region are summarized in Fig 12E, providing a numerical comparison that supports the visual improvements observed after filtering.

Wide-Depth Volumetric Imaging of Swimming Paramecia

One of the key advantages of holographic imaging is its ability to recover volumetric information in a plane-by-plane manner from a single recorded two-dimensional hologram. In this application, *HoloBio* was used to process a DLHM hologram [43] of three paramecia swimming in water at different axial positions within an inspection volume of approximately 2–3 mm^3.

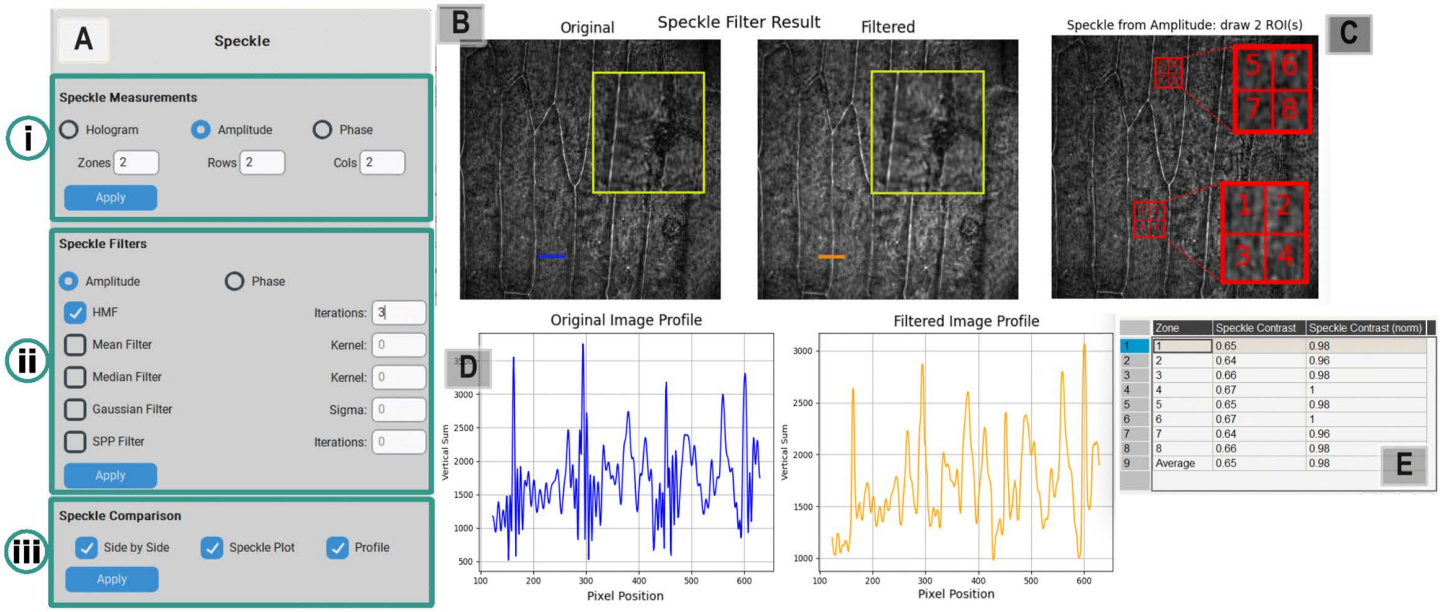

Fig 12. Speckle analysis and reduction workflow in HoloBio, illustrating an application of the Speckle Toolkit for a biological sample of onion cell tissue. (A) Interface is organized into three functional sections: Speckle Measurements, Speckle Filters, and Speckle Comparison. (B) Side-by-Side visualization of the original and filtered amplitude reconstructions (using the HMM filter, 3 iterations). (C) Selection of ROIs and subdivision into zones for speckle quantification. (D) Line profile comparison between original and filtered images.

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Fig 13A shows the *Offline* DLHM package interface for configuring reconstruction parameters and selecting the reconstruction algorithm. This panel allows manual adjustment of the propagation distance z, enabling each microorganism to be individually brought into focus. Fig 13 shows the reconstructed amplitude images (panels C-E) of the specimens from a recorded hologram (panel A) at three propagation distances. Reconstructed amplitude images were obtained using the KHDI method with the following parameters: wavelength $\lambda = 532\,nm$, square pixels with a side length of $6.9\,\mu m$, distances $L = 20\,mm$, and $z = 6.8\,mm$ (middle paramecium, panel C), $5.6\,mm$ (bottom paramecium, panel D), and $2.8\,mm$ (top paramecium, panel E). Red rectangles indicate zoomed regions of individual paramecia, obtained using the interactive zoom functionality integrated into *HoloBio*. To further improve visualization of the sample morphology, the Filters Toolkit was applied by selecting *Amplitude Viridis* option from the *Filters* menu in the *Tools* bar.

Wide-field cell tracking

This experiment was carried out using a previously recorded phase-reconstruction video of RBCs with a duration of *5* seconds at 15 fps. In *HoloBio*, particle tracking begins by uploading the video through **Load > Load Video** from the top control panel. Once loaded, playback can be managed using the **Play** and **Pause** buttons in the *Compensation Control* panel, which allows users to pause at the desired frame to initiate tracking. Before starting, several filters and parameters must be configured in the *Particle Tracking* panel, since the procedure relies on the Kalman algorithm [44]. The user may also activate the *World Coordinates* option to report tracking in real spatial units rather than pixel positions. In this example, the phase video

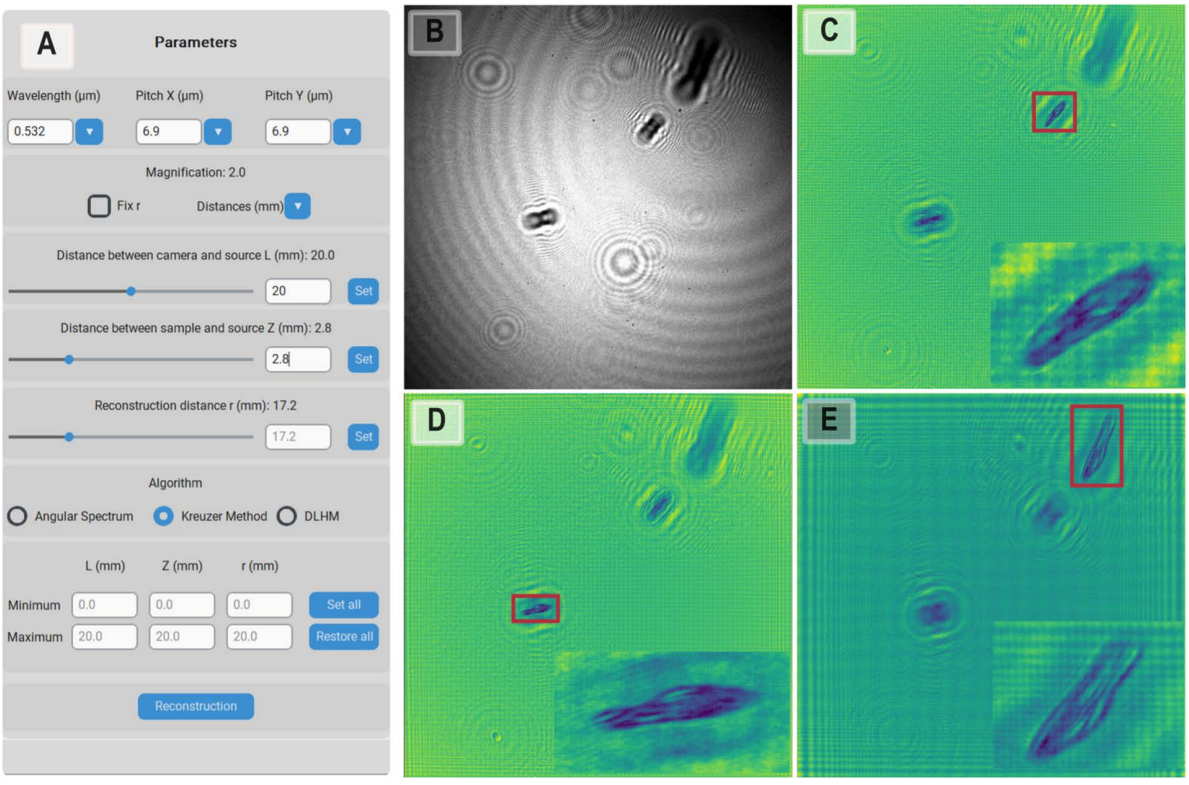

Fig 13. Offline DLHM reconstruction of paramecia in water using HoloBio. (A) Parameter panel of the Offline DLHM package, showing the configuration of reconstruction settings: wavelength $\lambda = 532\,nm$, pixel size $6.9\,\mu m$, source-to-camera distance $L = 20\,mm$. (B) Recorded in-line hologram of paramecia. (C–E) Reconstructed amplitude images at different propagation distances corresponding to individual paramecia. Red rectangles indicate regions zoomed using HoloBio's interactive zoom option.

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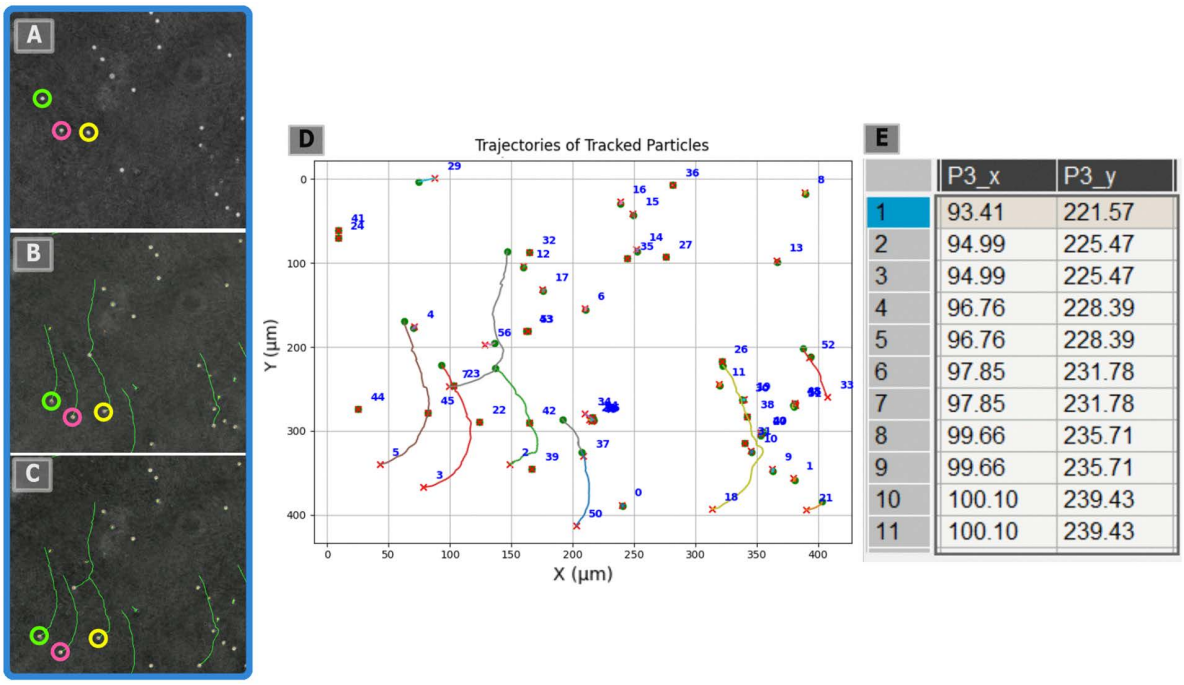
was acquired using an off-axis DHM system comprising a 10× microscope objective, and 3.75-μm pixel size digital camera. These parameters were input in the *Parameter* panel. Once all configurations are complete, clicking on the **Tracking** button initiates the analysis, which proceeds as illustrated in Fig 14A–C. These panels show successive frames where individual cells are detected and tracked. Fig 14D presents the estimated trajectories in the spatial domain with each path assigned to a unique color and index. Fig 14E displays the Positions Vector Table containing the frame number, elapsed time, and X–Y coordinates of each tracked sample, thus providing both qualitative visualization and quantitative data for further analysis.

Conclusions

This work presents ***HoloBio***, a free and open-source graphical user interface designed to facilitate the use of Digital Holographic Microscopy (DHM) and Digital Lensless Holographic Microscopy (DLHM) in biological imaging. Unlike existing libraries, plugins, or commercial platforms, *HoloBio* provides an integrated environment that unifies hologram acquisition, numerical reconstruction, and biologically oriented analysis tools within a single, intuitive software package. We demonstrated the versatility of *HoloBio* in addressing key tasks relevant to biological research. These included the reconstruction and numerical refocusing of red blood cell holograms, quantitative phase imaging and thickness estimation, cell detection and area measurements, speckle noise quantification and reduction in a complex onion cell tissue, and tracking of cells. A central contribution of *HoloBio* lies in its emphasis on accessibility and usability. By providing modular architecture optimized for real-time and offline processing, and by offering specialized DHM and DLHM packages, the software lowers the technical barriers associated with conventional coding-based libraries and fragmented plugins. Moreover, it has dedicated toolkits, including Bio-Analysis and Filters modules, equipping researchers with traditional functionalities tailored to biological and clinical applications.



	P3_x	P3_y
1	93.41	221.57
2	94.99	225.47
3	94.99	225.47
4	96.76	228.39
5	96.76	228.39
6	97.85	231.78
7	97.85	231.78
8	99.66	235.71
9	99.66	235.71
10	100.10	239.43
11	100.10	239.43

Fig 14. Cell tracking using the Offline DHM package in HoloBio. (A-C) Consecutive frames from the phase-reconstruction video showing object detection and tracking in real time. (D) Reconstructed trajectories of individual particles in the spatial domain, each displayed with a unique index and color. (E) Position Vector Table summarizing the tracking results, including frame number, elapsed time, and X–Y coordinates of each detected particle.

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HoloBio has the potential not only to accelerate the adoption of DHM and DLHM in the life sciences but also to foster reproducibility and innovation in quantitative phase imaging. In this work, HoloBio establishes a flexible and extensible foundation upon which future software enhancements can be built, including the integration of advanced machine-learning models for automated feature detection, support for three-dimensional reconstruction, and expansion toward multi-modal imaging scenarios. Additional development efforts will focus on incorporating GPU-accelerated numerical reconstruction to enable video-rate analysis for functionalities currently available only in offline mode. Furthermore, future updates aim to support real-time particle tracking directly from live holograms, extending HoloBio's capabilities for high-throughput and dynamic biological analysis.

Availability and Future Directions

HoloBio-GUI is an open-source platform available at https://github.com/SOPHIA-Research-Lab/HoloBio. Installation instructions and full documentation, including a user manual and video tutorials, are provided at https://sophia-research-lab.github.io/HoloBio/. Example holograms used in this work, along with the corresponding reconstruction parameters, are also made available on the same platform.

Currently, HoloBio supports two-dimensional (x–y) particle tracking. Future work will extend the platform to three-dimensional tracking by leveraging the intrinsic volumetric information encoded in digital holograms. By identifying the centers of defocused diffraction patterns and automatically estimating each cell's axial (z) position a future release of HoloBio will recover full 3D trajectories. This capability will enable analysis of particle dynamics through depth and provide a more comprehensive characterization of biological systems.

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Writing – review & editing: Carlos Trujillo, Ana Doblas, Raul Andres Castaneda Quintero.

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