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An orientation-independent DIC microscope allows high resolution imaging of epithelial cell migration and wound healing in a Cnidarian model

Jocelyn Malamy¹ and Michael Shribak^{2,*}

¹Department of Molecular Genetics and Cell Biology, The University of Chicago, 929 East 57th Street, Chicago, IL, 60657

²Marine Biological Laboratory, The University of Chicago, 7 MBL Street, Woods Hole, MA, 02543

Abstract

Epithelial cell dynamics can be difficult to study in intact animals or tissues. Here we use the medusa form of the hydrozoan *Clytia hemisphaerica*, which is covered with a monolayer of epithelial cells, to test the efficacy of an orientation-independent differential interference contrast (OI-DIC) microscope for *in vivo* imaging of wound healing. OI-DIC provides an unprecedented resolution phase image of epithelial cells closing a wound in a live, non-transgenic animal model. In particular, the OI-DIC microscope equipped with a 40×/0.75NA objective lens and using the illumination light with wavelength 546 nm demonstrated a resolution of 460 nm. The repair of individual cells, the adhesion of cells to close a gap, and the concomitant contraction of these cells during closure is clearly visualized.

INTRODUCTION

In the continual evolutionary arms race between the eaters and the eaten, animals have developed various types of the camouflage in order to be indistinguishable from the natural background: a hare changes fur color from white to gray, according to season, a leopard uses spotty camouflage to hide in the savannah, etc. The most fascinating examples of camouflage can be found in ocean creatures. For example, cuttlefish and squid alter their camouflage pattern according to the surrounding seabed. Some slowly moving animals living at the ocean surface, such as jellyfish, use their transparency to be invisible to their enemies (Johnsen, 2000). The transparency is a double-edged sword, providing both and advantage and a challenge to the researcher. The advantage is that light can penetrate the tissues, which makes them distinct from most intact animals. The challenge is that the researcher has to employ an interference microscopy to image the jellyfish.

One model that is gaining attention is the transparent marine animal, *Clytia hemisphaerica* (Houlston et al., 2010). *Clytia* is ideal for imaging epithelial cells because they occur as a flat squamous monolayer that covers the surface of the *Clytia* medusa. Epithelial cell sheets occur in all animals and are easily damaged by abrasion, surgery, or normal events such as

* Author for correspondence: mshribak@gmail.com, phone 1-508-289-7242.

the passage of food in the gut (Evans et al., 2013; Begnaud et al., 2016). Gaps must be rapidly closed to prevent infection and disease (Han and Ceilley, 2017). Therefore, understanding how cells in the epithelial sheet recognize and close a wound is of tremendous importance as a basic science question and for clinical applications. Epithelial cell dynamics are difficult to study in intact animals or tissues; therefore, much of our understanding of epithelial wound healing comes from artificially wounded monolayers of epithelial cells grown in tissue culture (reviewed in Begnaud et al., 2016). While epithelial cells are easy to visualize and manipulate in culture, it is important to develop *in vivo* models to complement the tissue culture approach.

The differential interference contrast (DIC) microscope is the most suitable instrument for imaging transparent animals (Shribak, 2012). However the contrast in conventional DIC imaging depends on the specimen orientation. The specimen under investigation has to be mechanically rotated to see all features. The major microscope manufacturers offer three types of DIC optics: high contrast/low resolution, intermediate contrast/intermediate resolution and low contrast/high resolution. The researcher has to choose what is most important in the image and what can be sacrificed. Recently the new quantitative orientation-independent (OI-) DIC microscope was proposed (Shribak and Inoué, 2006; Shribak, 2007; Shribak, 2009). OI-DIC provides high contrast/high resolution images with contrast independent of specimen orientation. Here we demonstrate how OI-DIC can reveal details of events during wound healing that are poorly resolved using standard DIC microscopy. Hence, OI-DIC is a valuable new tool for live imaging of dynamic cellular processes at high resolution, particularly where transparency of the tissue makes imaging a challenge.

RESULTS AND DISCUSSION

Observing wound healing in the medusa of *Clytia hemispherica* with a DIC microscope

The 2–3 week old medusa form of *Clytia* offers an exciting new way to examine cell movements during wound healing *in vivo*. The bell of the medusa is made up mostly of acellular mesoglea (jelly) (Houliston et al., 2010). The outside and inside of the bell is covered by a monolayer of epithelial cells. These layers provide an easily accessible, simple tissue for observation. The cells are squamous, hexagonal, and relatively large (50–100 μm in diameter, 5–10 μm in height). Gentle abrasion of the animal surface with a pulled glass pipette creates damage in the epithelial sheet. The cell sheet pulls apart to create a wound (Figures 1 and 2). Such wounds heal completely in 30–60 minutes (Figure 2). In addition, individual cells can become damaged, and this damage is repaired within minutes (see below).

The transparency of the epithelial cells and the underlying mesoglea render structures difficult to image using transmitted light. A differential interference contrast (DIC) microscope is a commonly used tool for imaging of unstained transparent live organisms, such as jellyfish, zebrafish, etc. and this approach reveals the cell outlines and the nuclei clearly (Figure 1, DIC). Other types of microscopy techniques do not work well with such specimens. Brightfield microscopy is practically useless (Figure 1, BF). A transparent large body can introduce significant wavefront aberration and light scattering into the probe beam in interferometric and holographic microscopes. It also destroys the mutual coherence

between probe and reference beams. Often the transparent animal body works as a lens, which smears an image of the condenser annulus on the objective phase ring in a phase contrast microscope. In this case the other techniques generate a weak image contrast or nothing. Their images have low optical resolution and optical sectioning. In order to mitigate the lens effect a live transparent specimen under investigation has to be squashed. In particular, Figure 1 (PhC) depicts the same region of a phase contrast image of a squashed jellyfish. The picture shows a clearly visible halo, which is an inherent artifact of the phase contrast method. We would like to mention that the image was taken with a positive phase contrast objective lens, where objects with a higher refractive index than the surrounding medium are displayed darker than objects with a lower refractive index. Currently the positive phase contrast is standard and widely available. However, the negative phase contrast objective lenses require a special order and are about twice expensive. An OI-DIC image of the same area is shown in Figure 1 (OI-DIC), where the objects with a higher refractive index than the surrounding medium are displayed brighter than objects with a lower refractive index. Two insets on the right display enlarged regions showing phase contrast and OI-DIC. In order to match a dependence of the image brightness on the refractive index we inverted the grayscale phase contrast image with using freely available software ImageJ (National Institutes of Health, <https://imagej.nih.gov>). As one can see the OI-DIC image is more sharp and clear. It offers higher contrast and thinner optical section.

Two interfering beams in a DIC microscope are sheared by about half of the resolution limit. Therefore both beams propagate along the same path and experience the same distortions, except a small submicroscopic volume in focus. Differential interference contrast microscopy reveals unlabeled tissues, organs, and other structures with high contrast and inherent optical sectioning, as only a thin region around the focal plane contributes coherently to image formation. A DIC microscope can employ high numerical aperture (NA) objective and condenser lenses and therefore provides best lateral resolution as well as best optical sectioning.

The OI-DIC microscope as a tool for imaging transparent live organisms

While DIC optics can reveal many new details of cellular behavior during epithelial wound healing (Kamran, Zellner, Kyriazis, Reynier, Kraus, and Malamy, submitted), it is still difficult to resolve the movements of lamellipodia, the adhesion of cells, or the events that occur to resolve damage within a cell. The contrast of DIC images depends on the orientation of specimen and the bias between interfering beams (Oldenbourg & Shribak, 2009). To overcome the limitations of DIC technique, a quantitative orientation-independent differential interference contrast (OI-DIC) microscope has been developed (Shribak & Inoué, 2006; Shribak, 2013; Shribak *et al.*, 2017). OI-DIC provides a novel approach to improving the resolution and the contrast of our images.

A schematic of the principle components of the OI-DIC microscope is shown in Figure 3. The set-up is based on an upright light microscope, which includes a halogen lamp with bandpass interference filter, crossed linear polarizer and analyzer, condenser and objective lenses, tube lens, and CCD camera. The microscope is transformed to the OI-DIC mode by adding two beam-shearing assemblies. The first assembly consists of pair of identical DIC

prisms **DIC1** and **DIC2**, and polarization rotator **PolRot1** sandwiched between the prisms. The shear directions of the DIC prisms are orthogonal. The first prism has the shear direction at 0° and the second prism has the shear direction at 90° . The polarization rotator works as a bi-stable element that rotates the beam polarization by 0° or 90° , called state OFF and state ON, accordingly. We employ a twisted-nematic (TN) liquid crystal cell as the 90° -polarization rotator. In operation the assembly shear direction can be oriented either at -45° or at 45° . Similarly, the second beam-shearing assembly consists of pair of identical DIC prisms **DIC3** and **DIC4**, and polarization rotator **PolRot2**. Orientation of the prisms **DIC3** and **DIC4** is 270° and 180° . The second assembly also includes a phase shifter, which allows to vary the bias between the two interfering beams. The phase shifter is an untwisted nematic liquid crystal cell, which works in the electrically controlled birefringence (ECB) mode. Its principal axis is oriented at 0° . Optical properties of the liquid crystal (LC) cells are determined by the amplitude of square wave voltage signals V_1 and V_2 , which are generated by LC-cell controller (signal generator). In particular, signal V_1 simultaneously switches the shear direction of assemblies, and signal V_2 varies the bias.

A desktop computer with custom written software sequentially establishes several settings of output signals of the LC-cell controller and takes the corresponding set of 4 or 6 raw DIC images from CCD camera. The images with orthogonal shear directions and different biases are processed according to a computation algorithm, detailed in the appendix. In the result we receive a quantitative image (map) of the optical path difference (OPD) gradient vector, which then integrated in order to get an OPD map. The OPD equals to the product of thickness and refractive index difference and represents the cell dry mass (Barer, 1952; Zangle & Teitell, 2014). The obtained OPD images can be enhanced by using deconvolution or the inverse Riesz transform (Larkin & Fletcher, 2014; Shribak *et al.*, 2017).

We implemented OI-DIC set-up on upright microscope Olympus BX61 (Olympus, Tokyo, Japan) (Figure 4). The microscope was equipped with 100W halogen lamp, band-pass interference filter 546/10 nm, revolving nosepiece U-P4RE, objective lens UPlan FI 40 $\times$ /0.75P, condenser DICD, condenser lens U-TLD, and video camera adapter U-TV0.5 $\times$ C-3. The images were captured by a monochromatic CCD camera Infinity 3-1M (Lumenera, Ottawa, Canada). We purchased two sets of standard DIC sliders U-DICTHR and U-DIC40HR from the microscope manufacturer. The high-resolution objective side DIC slider U-DICTHR has the smallest shear angle of $39\mu\text{rad}$ (Shribak, 2013). A condenser side DIC slider U-DIC40HR is its match when a 40 $\times$ objective lens and U-TLD condenser lens are used. Then the optical prisms were taken out from the original sliders. The crossed pairs of bare DIC prisms and liquid crystal cells were placed into two custom-made holders. We call the units as “the beam shearing assemblies”. Thus, the standard DIC sliders were replaced by new compact beam shearing DIC assemblies. Because dimensions of the DIC assemblies are exactly the same as dimensions of the corresponding DIC prisms the microscope does not require a custom modification. The first assembly (condenser-side) includes two DIC prisms U-DIC40HR and TN liquid crystal cell (Arcoptix, Neuchatel, Switzerland). The second assembly (objective-side) consists of two DIC prisms U-DICTHR, TN liquid crystal cell and ECB liquid crystal cell (Bolder Vision Optik, Boulder, CO, USA). The liquid crystal cells were connected to two outputs of the USB LC-cell controller (Arcoptix, Neuchatel,

Switzerland). Each output supplies a square wave signal with frequency of 1.6 KHz and variable voltage from 0 to 10V. Using MATLAB (The MathWorks, Inc., Natick, MA, USA), we developed custom software for setup control and image processing. If necessary, the software and reasonable technical help are available from Shribak's lab (mshribak@gmail.com). A description of one of the OI-DIC algorithms can be found in Appendix.

Figure 1 (DIC, OI-DIC) shows an example of conventional and orientation independent DIC images of the same field. The shear direction in the conventional DIC picture is vertical. Therefore the vertical lamellipodia (A) is hardly visible in the conventional image and it is clear visible in the OI-DIC image. The horizontal lamellipodia (B) is visible in the both images. In addition, the OI-DIC corrects a non-uniformity of illumination, which is present in the conventional image. The brightness of the conventional DIC image depends non-linearly on optical path difference, bias, shear amount and shear direction. The brightness of the OI-DIC image is proportional to the optical path difference only. In principle, the OI-DIC image brightness can be color coded in order to provide a useful way to detect subtle aspects of cell behavior in a living system.

For verification of the resolution, we carried out several experiments using artificial and biological specimens. In particular, we investigated a man-made specimen: a phase test target developed by the Marine Biological Laboratory in Woods Hole, Massachusetts in collaboration with the National Nanofabrication Facility at Cornell University (Oldenbourg *et al.*, 1996). The specimen has a 90 nm thick SiO₂ film deposited on a 170 μ m thick glass coverslip. The film was treated to remove the SiO₂ from regions exposed to the electron beam by microlithography. The target includes a 75 μ m diameter Siemens Star that consists of 36 wedge pairs. The period near the outer edge is 6.5 μ m, decreasing linearly toward the center. The coverslip was mounted on a 1 mm thick regular microscope slide. The micro-relief structure was placed on the side of the coverslip nearest the slide. A gap between microscope slide and coverslip was filled with distilled water.

We also imaged amplitude Siemens Star test target developed by Changhuei Yang and Daniel Martin (Caltech, Pasadena, CA, USA). The Siemens Star pattern was fabricated in a 100 nm thick layer of gold on a microscope slide by focused ion beam milling. The microscope slide was covered by a 170 μ m thick glass coverslip. The outer circle radius is 23 microns and the inner circle radius is 1 micron. Since there are 36 pairs of rays on the star, this will produce a periodicity ranging from ~175nm at the inner circle edge to 4 μ m on the outer circle edge ($2\pi R/36$), where R is radius. The amplitude Siemens Star test was imaged in bright-field mode. The OI-DIC assemblies were removed.

The both, OI-DIC and bright-field images, are displayed in Figure 5a. The left part of the montage represents the bright-field image. The right part shows the OI-DIC map, which was processed according to the inverse Riesz transform. The inverse Riesz transform image can be considered to be an OPD image that has been edge enhanced. The enhancement is equivalent to a high-pass image processing filter. The inverse Riesz transform does not include a deconvolution. The resolution measure used is not affected by the Riesz transform. We used Olympus 40 $\times$ objective lens with 0.75NA and light with wavelength 546nm and

bandwidth 10 nm. In order to decrease the depolarization of light and achieve a high interference contrast the numerical aperture of illuminating beam was restricted to 0.5. For estimation of theoretical resolution, which takes into account objective and condenser apertures, NA_{obj} and NA_{cond} , we used the resolution equation derived by Hopkins & Barham (Hopkins & Barham, 1950; Born & Wolf, 1999; Oldenbourg & Shribak, 2010). They applied the Rayleigh criterion for resolving two pinholes that are equally bright. According to the equation $\delta = L(m)\lambda/NA_{obj}$, where $m = NA_{cond}/NA_{obj}$. If $m=0.67$ then $L=0.68$. In our case a theoretical resolution limit of the microscope δ was 495 nm.

The grating period the both Siemens Stars is decreases continuously towards the smallest period of ~ 100 nm at the inner circle edge. Using a profile plot function in ImageJ software (<https://imagej.nih.gov/ij/>) we measured the intensity along medians of a bright and dark wedge pair, $I_b(R)$ and $I_d(R)$, where R is radius. The wedge medians are depicted by dash-and-dot lines m_b and m_d . The contrast was computed by the following formula:

$$C = \frac{I_b(R) - I_d(R)}{I_b(R) + I_d(R)}.$$

In order to suppress the contribution from dusts and defects we averaged the contrast data of three wedge pairs. Figure 5b displays dependences of the contrast in the bright-field and the OI-DIC images on the grating period p , where $p = 2\pi R/36$. We applied the 2%-threshold (dashed horizontal line in Fig. 5b) in order to define when the modulation signal in the plot started to disappear. The star pattern became unresolved at period of 460 nm in the OI-DIC image and at period of 590 nm in the bright-field image.

The theoretical resolution limit of 495 nm was computed for ideal conditions. But the real microscope resolution is also affected by the wavefront aberrations of objective lens. We did not select our objective lens for the minimum of wavefront aberrations. As one can see, the resolution of bright-field image is reduced by $\sim 16\%$ in comparison with the theoretical limit. However the optical image subtraction and the computation procedure in OI-DIC suppress the resolution degradation due to small wavefront aberrations. In the result, resolution of the OI-DIC exceeds slightly the theoretical limit. Images of gratings on the right to the Siemens Star also confirm that resolution of the OI-DIC image is less than 500 nm.

The OI-DIC microscope as a tool for live imaging of wound healing

Numerous wound healing studies in tissue culture cells and in embryos have revealed that, in many cases, lamellipodia are produced by cells at the wound margin, and these cells migrate into the wound area (reviewed in Bagnaud et al., 2014). When the edges of lamellipodia meet across the gap they establish cell-cell junctions and restore the integrity of the epithelial cell sheet. Wounds within a single cell are either resolved by the sheet extruding the remains of that cell, or by repair of the cell (Fenteany et al., 2000; Evans et al., 2013; Bagnaud et al., 2016).

Using OI-DIC, we created time-lapse movies of small epithelial wounds healing in *Clytia* (Movies 1–3). The time interval between frames was 13 seconds. These movies also contrast

the image quality between standard DIC and OI-DIC. Still OI-DIC and standard DIC images from the time lapse experiments are shown in Figures 6–8.

Wounds healed by extension of lamellipodia from cells at the wound margin. Contact between the edges of lamellipodia is followed by a “zippering” together of cells on opposite sides of the gap, followed rapidly by contraction that appeared to pull the wound closed at the regions of cell-cell contact (Figure 6, Movie 1). While lamellipodia are easily visualized by standard DIC, OI-DIC makes it much easier to detect the ruffling activity and the overlap of lamellipodia. Circular gaps healed similarly, with large lamellipodia meeting across the gap to create new cell-cell adhesions, and a concomitant contraction altering the shape of the marginal cells (Figure 7, Movie 2). Again, cell outlines and the dynamic events that occurred during wound closure were much better visualized with OI-DIC.

These images are arguably the best resolution ever presented for wound healing in epithelial cells in an intact animal. It allowed us to see the authentic responses of the animal to wounding in the complex context of intact tissue, and to dissect responses to wounds of irregular shapes, which are indeed the most likely to occur in nature.

Healing of an injury to single cells

OI-DIC allowed us to follow the fate of several badly damaged cells in detail over time. For example, in Figure 8 a cell and several cell pieces have become stretched across a wound area and all appear to be badly shredded. The larger cell repairs while one of the cell pieces extends lamellipodia and appears to merge with neighboring cells (Figure 8, Movie 3). The cell outlines that are apparent with OI-DIC make it possible to decipher these complex events. This type of cellular healing has not in the past been observed at this resolution, to our knowledge.

The ability to see cells in great detail as they move in an intact animal during wound repair is an exciting step forward in this field. The use of OI-DIC will allow a detailed characterization of processes not previously observed. The experimental set up is such that, in the future, epithelial cells could be easily exposed to chemical inhibitors, such as RHO-GTPase inhibitors or cytoskeletal inhibitors, and the resulting change in cell dynamics could be studied at high resolution with OI-DIC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Barer R. Interference microscopy and mass determination. *Nature*. 1952; 169:366–367. [PubMed: 14919571]
- Begnaud S, Chen T, Delacour D, Mège RM, Ladoux B. Mechanics of epithelial tissues during gap closure. *Curr Opin Cell Biol*. 2016; 42:52–62. [PubMed: 27131272]
- Biggs D, Andrews M. Acceleration of iterative image restoration algorithms. *Appl Opt*. 1997; 36:1766–1775. [PubMed: 18250863]
- Born, M., Wolf, E. *Principles of Optics: Electromagnetic Theory of Propagation, Interference and Diffraction of Light*. Cambridge University Press; Cambridge: 1999.
- Evans ND, Oreffo ROC, Healy E, Thurnera PJ, Mana YH. Epithelial mechanobiology, skin wound healing, and the stem cell niche. *Journal of the Mechanical Behavior of Biomedical Materials*. 2013; 28:397–409. [PubMed: 23746929]
- Fenteany G, Janmey PA, Stossel TP. Signaling pathways and cell mechanics involved in wound closure by epithelial cell sheets. *Curr Biol*. 2000; 10:831–8. [PubMed: 10899000]
- Han G, Ceilley R. Chronic Wound Heling: A review of current management and treatments. *Adv Therapy*. 2017; doi: 10.1007/s12325-017-0748-y
- Hopkins HH, Barham PM. The influence of the condenser on microscopic resolution. *Proceedings of the Physical Society of London*. 1950; 63 B:737–744.
- Houliston E, Momose T, Manuel M. *Clytia hemisphaerica*: a jellyfish cousin joins the laboratory. *Trends Genet*. 2010; 26:159–67. [PubMed: 20227783]
- Johnsen S. Transparent animals. *Scientific American*. 2000; 282(2):80–89.
- Larkin KG, Fletcher PA. Isotropic scalar image visualization of vector differential image data using the inverse Riesz transform. *Biomed Opt Express*. 2014; 5:907–920. [PubMed: 24688823]
- Oldenbourg, R., Inoué, S., Tiberio, R., Stemmer, A., Mei, G., Skvarla, M. Standard test targets for high resolution light microscopy. In: Hoch, HC, Jelinski, LW., Craighead, HG., editors. *Nanofabrication and Biosystems*. Cambridge University Press; New York: 1996. p. 123–138.
- Oldenbourg, R., Shribak, M. Microscopes. In: Bass, Michael, editor. *Handbook of Optics, Third Edition, Vol. 1: Geometrical and Physical Optics, Polarized Light, Components and Instruments*. McGraw-Hill; 2009.
- Shribak M, Inoué S. Orientation-Independent Differential Interference Contrast Microscopy. *Applied Optics*. 2006; 45(3):460–469. [PubMed: 16463729]
- Shribak, M. Orientation-independent differential interference contrast microscopy technique and device. US Patent. 7233434. 2007.
- Shribak, M. Orientation-independent differential interference contrast microscopy technique and device. US Patent. 7564618. 2009.
- Shribak, M. Differential interference contrast microscopy. In: Shaked, NT, Zalevsky, Z., Satterwhite, LL., editors. *Biomedical Optical Phase Microscopy and Nanoscopy*. Elsevier; 2012.
- Shribak M. Quantitative orientation-independent DIC microscope with fast switching shear direction and bias modulation. *J Opt Soc Am A*. 2013; 30:769–782.
- Shribak M, Larkin K, Biggs D. Mapping of optical path length and image enhancement using orientation-independent differential interference contrast microscopy. *Journal of Biomedical Optics*. 2017; 22(1):16006. [PubMed: 28060991]
- Sonnemann KJ, Bement WM. Wound repair: toward understanding and integration of single-cell and multicellular wound responses. *Annu Rev Cell Dev Biol*. 2011; 27:237–63. [PubMed: 21721944]
- Zangle TA, Teitell MA. Live-cell mass profiling: an emerging approach in quantitative biophysics. *Nature methods*. 2014; 11(12):1221–1228. [PubMed: 25423019]

APPENDIX

For convenience of the reader we briefly explain one of the image processing algorithms, which we are using. This and other algorithms have been previously described elsewhere

(Shribak & Inoué, 2006; Shribak, 2013). The intensity distribution in the DIC image $I(x, y)$ can be described by using a model of interference of two overlapping identical coherent images with OPD $\phi(x, y)$, slightly offset from each other by distance d along direction σ .

$$I(x, y) = \tilde{I} \sin^2 \left\{ \frac{\pi}{\lambda} [\Gamma + \sqrt{2} d \gamma(x, y) \cos (\theta(x, y) - \sigma)] \right\},$$

where $\tilde{I}$ is the initial beam intensity, λ is wavelength, Γ is bias, $\gamma(x, y)$ is the magnitude and $\theta(x, y)$ is the azimuth of OPD gradient vector, respectively.

In order to find the optical path difference gradient magnitude $\gamma(x, y)$ and azimuth $\theta(x, y)$ the OI-DIC microscope varies the direction of image displacement *and* bias, σ and Γ . We capture two sets of raw DIC images at shear directions -45° and $+45^\circ$ with negative and positive biases: $-\Gamma_0$, 0, and $+\Gamma_0$. The following group of equations represents these six DIC images:

$$I_{i,j}(x, y) = \tilde{I} \sin^2 \left\{ \frac{\pi}{\lambda} \left[j\Gamma_0 + \sqrt{2} d \gamma(x, y) \cos \left(\theta(x, y) - (-1)^j \frac{\pi}{4} \right) \right] \right\},$$

where $j = -1, 0, 1$.

Initially two terms are computed ($i = 1, 2$):

$$A_i(x, y) = \frac{I_{i,1}(x, y) - I_{i,-1}(x, y)}{I_{i,1}(x, y) + I_{i,-1}(x, y) - 2I_{i,0}(x, y)} \tan \left(\frac{\pi\Gamma_0}{\lambda} \right).$$

Using the obtained terms we can calculate the quantitative two-dimension distributions (map) of the gradient magnitude and azimuth of OPD in the specimen as:

$$\gamma(x, y) = \frac{\lambda}{2\sqrt{2}\pi d} \sqrt{\sum_{i=1}^2 \arctan^2 [A_i(x, y)]},$$

$$\theta(x, y) = \arctan \left(\frac{\arctan A_2(x, y)}{\arctan A_1(x, y)} \right) - \frac{\pi}{4}.$$

The gradient magnitude represents increment of the optical path difference, which is in nanometers, along lateral coordinate, which is also in nanometers. Thus, the gradient magnitude is unitless.

Then we use the 2-dimensional Fourier integration of obtained OPD gradient vector map to compute a map of the optical path difference $\phi(x, y)$. (Shribak *et al.*, 2017). For this purpose the OPD gradient vector $\mathbf{G}(x, y)$ can be presented as a complex number:

$$\mathbf{G}(x, y) = \gamma(x, y) e^{i\theta(x, y)},$$

where real and imaginary parts are X- and Y- components of the OPD gradient vector, respectively.

At first we apply the 2-dimensional Fourier transform to the left and the middle parts of the equation above. Then the resultant integral equation can be solved by partial integration. After using the inverse 2-dimensional Fourier transform we receive the following formula for computation of the optical path difference $\phi(x,y)$:

$$\phi(x,y) = F^{-1} \left[\frac{F[G(x,y)]}{i(\omega_x + i\omega_y)} \right],$$

where ω_x and ω_y are spatial angular frequencies.

Taking into account the right part of equation, finally we get formula for computing the OPD map:

$$\phi(x,y) = F^{-1} \left[\frac{F[\gamma(x,y)e^{i\theta(x,y)}]}{i(\omega_x + i\omega_y)} \right].$$

The resolution of the OPD map can be further enhanced by applying a 2-dimensional deconvolution algorithm to correct for the optical imaging limitation of the microscope and reduce the out-of-focus haze (Biggs & Andrews, 1997). The used deconvolution technique was developed by D. Biggs (KB Imaging Solution LLC, Loomis, CA, USA). In many situations, the exact mapping of the OPL is not essential, and more visually informative rendering is desired. In this case we apply the inverse Riesz transform (Larkin & Fletcher, 2014), which was developed for us by K. Larkin (Nontrivialzeros Research, Putney, Australia).

Lay description

In this paper, we show how a newly developed microscopic technique, orientation-independent differential interference contrast (OI-DIC), can give us an unprecedented look at dynamic cell behavior in a transparent tissue. As an example of the power of the new microscope to address biological questions, we used it to examine wound healing in the small, transparent jellyfish *Clytia hemisphaerica*. *Clytia* is covered with a single layer of epithelial cells. Upon wounding, the epithelial cells migrate inwards from the margins of a wound to permanently close it. The process looks remarkably like processes that occur in vertebrates such as ourselves, suggesting that insights gained in *Clytia* wound healing may have a far reaching implications to basic science questions and even to the treatment of wounds in the clinic. The OI-DIC microscope provides halo-free phase images of live wound healing at the highest resolution, allowing the detection of details and events not visible using standard DIC or phase contrast microscopy. These results show that OI-DIC microscopy is an important new tool for studying cells and cell dynamics *in vivo*.

Figure 1.

Epithelial wound in a 3 week old *Clytia* medusa visualized with diverse microscopic methods. Top Panels: *Clytia* viewed from the top (left) and side (right). Middle and Bottom Panels: An example of the same field of *Clytia* with a healing epithelial wound imaged by brightfield (BF), phase contrast (PhC), conventional DIC (DIC) and orientation independent DIC (OI-DIC) techniques. The brightfield image has extremely low contrast. The phase contrast picture shows a clearly visible halo, which is indicated by arrows. The shear direction in the conventional DIC picture is vertical. The vertical lamellipodia A is hardly visible in the conventional image and it is clearly visible in the OI-DIC image. The horizontal lamellipodia B is clearly visible in the both images. The grayscale phase contrast image is inverted in order correspond the OI-DIC image.

Figure 2. Epithelial wound healing is rapid. Standard DIC microscopy reveals an irregular wound that heals in approximately an hour, leaving no scar.

Figure 3.
Principal schematic of the OI-DIC microscope.

Figure 4. OI-DIC components installed on upright microscope Olympus BX61. (1) Condenser-side beam-shearing DIC assembly; (2) objective-side beam-shearing DIC assembly; (3) Arcoptix USB LC-cell controller; (4) Lumenera monochromatic CCD camera.

(a)

(b)

Figure 5.

(a) OI-DIC and bright-field images of phase and amplitude Siemens Star resolution test targets. (b) Dependences of the contrast in the bright-field and the OI-DIC images on the grating period.

Figure 6. OI-DIC microscopy allows enhanced imaging of the cells in a healing epithelial wound. The ruffling lamellipodia of migrating cells are clear (arrows in 4 min panel), and one can visualize the joining together of cells across the wound gap. See Movie 1 for more a more detailed view.

Figure 7. OI-DIC microscopy allows the visualization of a small circular wound by a combination of lamellipodia and “purse string” contraction of the cell edges at the wound’s interior. Note the changes in shape of the cells as they close the wound. See Movie 2 for a more detailed view of the process.

Figure 8. OI-DIC microscopy shows healing of a single damaged cell in under 10 minutes. See Movie 3 for a more detailed view of the process.