Focus plane detection criteria in digital holography microscopy by amplitude analysis

Frank Dubois, Cédric Schockaert, Natacha Callens and Catherine Yourassowsky

Université Libre de Bruxelles Microgravity Research Center 50 Av. F. Roosevelt, CP 165/62 B-1050 Brussels (Belgium)
frdubois@ulb.ac.be

Abstract: We propose and test a focus plane determination method that computes the digital refocus distance of an object investigated by digital holographic microscopy working in transmission. For this purpose we analyze the integrated amplitude modulus as a function of the digital holographic reconstruction distance. It is shown that when the focus distance is reached, the integrated amplitude is minimum for pure amplitude object and maximum for pure phase object. After a theoretical analysis, the method is demonstrated on actual digital holograms for the refocusing of pure amplitude and of pure phase microscopic samples.

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References and links

1. Introduction

Digital holographic microscopy (DHM) provides a numerical investigation of the third dimension by performing a plane-by-plane refocusing that overcomes the small depths of focus due to the high numerical apertures of the microscope lenses and the high magnification ratios [1-4]. The digital hologram recorded by a CCD camera is processed to compute the phase and the intensity information. The complex amplitude signal, obtained from this two information, is then propagated by a discrete implementation of the Kirchhoff-Fresnel equation to perform a digital holographic refocusing of the sample image, slice by slice [4, 5].

Digital holographic microscopy has been demonstrated in many applications as in refractometry [6], observation of biological samples [7-11], living cells analysis [12-16] and velocimetry [17]. Due to its digital nature, this technique allows to implement powerful
processing to improve the digital holographic reconstruction [18], to control the image size as a function of refocusing the distance and the wavelength [19], to perform 3D pattern recognition [20-22], to process the border artifacts [23,24], to implement twin-image noise elimination techniques [25,26] and to perform quantitative phase contrast imaging [5,13,14,18] and aberration compensation [27,28].

Digital holography provides a tool to refocus an object. However, it does not provide any criterion when the best focus distance is reached. Indeed, if digital holographic reconstruction can refocus a sample slice-by-slice as the focusing stage of a classical imaging system, the refocusing of an object has to be determined by an external criterion. In that way, a refocus criterion based on a gradient computation has been investigated [29]. This approach can be powerful when the objects under investigation give rise to sharp images at the best refocus distance. A sharpness metric based on the self-entropy has been described [30]. It has been also proposed to use a criterion based on the maximization of the intensity local variance [31]. Ferraro and al. proposed a method where an operator focuses the object under test and the amount of focus change in time is obtained by measuring its phase change [32]. The defocus distance is then introduced to digitally refocus the object. Recently, the theory of Fresnelet has been applied to compute a new sharpness metric related to the sparsity of the wavelet coefficients and their energies [33]. This method is however computationally time consuming.

In this article we propose a focus determination methodology that takes benefit of the invariance of both energy and amplitude integration. Those invariance properties allow to build two focus criteria, respectively for pure amplitude and for pure phase objects, that are based on the score of the integrated amplitude modulus. In section 2, we describe the theoretical aspects and we derive the focus criteria for the both types of objects. Although the general case concerns objects that have mixed amplitude and phase modulations, the pure amplitude or phase cases are of considerable practical importance in microscopy. For examples, biological cells are mostly transparent and can be considered as pure phase objects while, for 3D velocimetry applications, the objects are very often opaque particles. The section 3 is devoted to the experimental demonstrations on actual digital holograms. Conclusions and perspective are given in section 4.

2. Theoretical aspects

2.1 Digital holography background and conservation of the integrated amplitude

Assuming a beam propagating in the z-axis direction, the digital holographic reconstruction process consists in computing the complex amplitude \( v_d(x', y') \) in a plane \( P' \) perpendicular to the z-axis knowing the complex amplitude \( u_0(x, y) \) in a plane \( P \) parallel to \( P' \) and separated from it by a distance \( d \). The spatial coordinates \((x, y)\) and \((x', y')\) are in the plane \( P \) and \( P' \). The relationship between \( v_d(x', y') \) and \( u_0(x, y) \) is given by the Kirchhoff-Fresnel equation that we assume paraxial:

\[
v_d(x', y') = \exp(\text{i}kdF^{-1}(c_{x'},y') \exp\left(-\frac{jkd}{2} (\nu_x^2 + \nu_y^2)\right)F^+_{(c_{x'},y')}u_0(x, y)
\]

Where \( \lambda \) is the wavelength, \( k=2\pi/\lambda \), \( (\nu_x, \nu_y) \) are the spatial frequencies, \( j^2 = -1 \), and \( F^+_{(c_{x'},y')}g(\alpha, \beta) \) denotes the direct or inverse 2D continuous Fourier transformations defined by:

\[
F^+_{(c_{x'},y')}g(\alpha, \beta) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \exp\left(\pm 2j\pi(\alpha\eta + \beta\xi)\right)g(\alpha, \beta)d\alpha d\beta
\]
As we can assume that the factor $\exp(jkd)$ at the right hand side of Eq. (1) does not play any significant role for the digital holographic refocusing, we define the effective digital holographic reconstructed or propagated amplitude $u_d(x', y')$ in $P'$ by:

$$u_d(x', y') = F_{(C)x', y'}^{-1} \exp \left( - \frac{jkd\lambda^2}{2} (v_x^2 + v_y^2) \right) F_{(C)x, y}^{+1} u_0(x, y)$$

(3)

By taking the Fourier transform of Eq. (3) and by computing the squared modulus, we obtain that the power spectrum $|U_d(v_x, v_y)|^2$ of $u_d(x', y')$ is equal to the power spectrum $|U_0(v_x, v_y)|^2$ of $u_0(x, y)$:

$$|U_d(v_x, v_y)|^2 = |U_0(v_x, v_y)|^2$$

(4)

Equation (4) means that the information is preserved by the digital holographic propagation of Eq. (3). Therefore the information content is insensitive to any refocus change by digital holography. This situation is different with respect to the classical imaging systems using incoherent lighting. Indeed, with such an imaging system, a defocus provides a loss of the image sharpness in a low-pass filtering process that modifies the shape of the power spectrum. As a consequence, there is no way to find a refocusing criterion based on the only digital holographic signal and it is necessary to add a criterion to determine the focusing distance of an object.

We consider some invariant properties of the optical field under the propagation defined by Eq. (3). The energy $E$ is invariant:

$$\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} |u_d(x', y')|^2 dx' dy' = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} |u_0(x, y)|^2 dx dy = E$$

(5)

The integral in the $xy$ directions of the complex amplitude is also invariant: Consider that we propagate the complex amplitude field equal to $u_0(x, y) + A$, where $A$ is an arbitrary amplitude value constant in the $(x,y)$ plane. The energy conservation leads to:

$$\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} |u_d|^2 + |A|^2 + A^* u_0 + A u_0^* dx' dy' = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} |u_d|^2 + |A|^2 + A^* u_d + A u_d^* dx dy$$

(6)

where we used the fact that the constant amplitude $A$ is invariant under the propagation defined by Eq. (3), and where we omitted, for conciseness, the explicit spatial dependency of $u_0(x, y)$ and $u_d(x, y)$. From Eq. (6), we have:

$$\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} (A^* u_0 + A u_0^*) dx' dy' = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} (A^* u_d + A u_d^*) dx dy$$

(7)

By combining the Eq. (7) respectively obtained with pure real and pure imaginary $A$ values, we can conclude that:
\[ \int \int u_d(x', y') dx' dy' = \int \int u_0(x, y) dx dy = B \tag{8} \]

Equation (8) means that \(B\), the integral of the amplitude in the \(xy\) directions, is invariant with respect to the digital holographic propagation. It results that the modulus of \(B\) is also an invariant. Therefore, using the usual Cauchy-Schwartz inequality relation, we obtain:

\[ |B| \leq \int \int |u_d(x, y)| dx dy = M_d \tag{9} \]

Equation (9) indicates that the integral \(M_d\) of the amplitude modulus has a global lower bound which is independent of \(d\).

2.2 Focus criterion for pure amplitude objects

We consider a pure amplitude object located in a plane. We will show that its image is refocused at distance \(d\) when the integral defined in Eq. (9) is minimum. The refocus distance is \(d\) if:

\[ \int \int |u_d(x, y)| dx dy \text{ is minimum} \tag{10} \]

Indeed, consider first an object that is a real positive transparency \(t(x, y)\) illuminated by a constant real positive amplitude \(C\). We have in the focus plane:

\[ u_d(x, y) = t(x, y)C \tag{11} \]

As \(u_d(x, y)\) is real positive valued, it results that:

\[ \int \int u_d(x, y) dx dy = \int \int \left| u_d(x, y) \right| dx dy = \int \int \left| u_d(x, y) \right| dx dy \tag{12} \]

We denote the complex amplitude in an out of focus plane by \(u_d(x', y')\). By invoking the invariance of the integrated amplitude, we obtain:

\[ \left| \int \int u_d(x, y) dx dy \right| = \left| \int \int u_d'(x', y') dx' dy' \right| \tag{13} \]

As \(u_d(x', y')\) is not the amplitude in the focus plane, it is \textit{a priori} complex and the second equality of Eq. (12) has to be replaced by:

\[ \left| \int \int u_d(x', y') dx' dy' \right| \leq \int \int \left| u_d(x', y') \right| dx' dy' \tag{14} \]
By combining Eq. (12), Eq. (13) and Eq. (14):

\[
\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} |u_d(x, y)| dx dy \leq \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} |u'_d(x', y')| dx' dy'
\]  

Equation (15) expresses that the integral of the amplitude modulus is minimized when the focused plane is reached. When we consider the same object illuminated by a tilted plane wave, the amplitude modulus pattern reconstructed by digital holography remains identical except that there is a small shift in its position with respect to the un-tilted illumination case. Therefore, the integration of the amplitude modulus of the diffraction pattern remains identical to the one of the un-tilted case and the minimum of the modulus integration occurs also at the refocusing distance \(d\). In most of the practical cases of interest, even in the case of non-plane wave illumination, the objects are small enough to be considered as illuminated by a plane wave.

Consider the example of the real amplitude object \(u_o(x, y)\) showed by the Fig. 1(a). It represents a clear disk in a screen back-illuminated by a constant phase plane wave. When the object is out of focus by a distance \(d\), diffraction effects are appearing with the typical oscillations shown by the Fig. 1(b).

Figures 1(c) and 1(d) represent the horizontal amplitude profiles across the center of the patterns of, respectively, Fig. 1(a) and Fig. 1(b). As the diffraction pattern presents positive and negative regions [Fig. 1(d)] while the un-diffracted one [Fig. 1(c)] has only a positive region, it results that the integration of the modulus \(|u_d(x, y)|\) will be minimized when \(d=0\).
This example is an illustration of the optical field behavior that is constrained to fulfill the invariance of the integrated intensity and amplitude. The refocus criterion is based on this phenomenon.

2.3 *Focus criterion for pure phase objects*

We consider now the case of pure phase objects. We assume that a pure phase object is illuminated in transmission by a plane wave that is propagating along the z axis and that the physical thickness of the object is small in comparison with the depth of focus of the imaging system. As in the amplitude case, we assume that the object is located at a distance $d$ with
respect to the focus plane of the imaging system. The beam emerging out of the object is expressed by:

\[ u_d(x, y) = a \exp\{j\phi(x, y)\} \quad (16) \]

Where \( \phi(x, y) \) is the optical phase change introduced by the phase object and \( a \) is the uniform real amplitude of a illuminating plane wave. As we consider a pure phase object, the emerging intensity is constant when the focus image of the object is reached. Indeed the intensity image of the object disappears from the focus image as it is well known in optical microscopy. It results also that the modulus of the amplitude is constant in the focus plane. When the image of the object is defocused, the refraction created by the object’s phase modulates the amplitude and the resulting intensity. As a consequence the defocus is at the origin of fluctuations of the amplitude modulus and the intensity. As in the previous case, the invariant energy and amplitude integral are fulfilled. We will show that its image is refocused at distance \( d \) when the integral defined in Eq. (9) is maximum. The refocus distance is \( d \) if:

\[ \int \int \int \int u_d(x, y) dx dy \quad \text{is maximum} \quad (17) \]

To understand how this criteria is operating, consider a collection of \( N \) sampled amplitudes that are obtained from a pure phase object, not necessarily focus, that is illuminated in transmission by a plane wave of amplitude \( a \). For the purpose of the demonstration, there is no need to consider the explicit 2D nature of the optical field and we can consider only one discrete spatial variable \( s \). We have:

\[ u_s = a_s \exp\{j\phi_s\} \quad (18) \]

Where \( s = 0, ..., N-1 \), \( a_s \) is the amplitude modulus and \( \phi_s \) the optical phase at the sampling point \( s \).

The energy conservation is expressed by:

\[ E = \sum_{s=0}^{N-1} a_s^2 = N a^2 \quad (19) \]

The focus metric defines by Eq. (9) becomes with the sampled signal:

\[ M_d = \sum_{s=0}^{N-1} a_s \quad (20) \]

When the image of the object is focus, all the amplitudes \( a_s \) are equal to \( a \). To demonstrate that \( M_d \) is maximum when all \( a_s = a \), we consider the problem that consists to look at the \( a_s \) that maximizes \( M_d \) under the constraint of a constant energy \( E \). The Lagrange multipliers method solves this problem. We form the function \( f(a_s) \):

\[ f(a_s) = \sum_{s=0}^{N-1} a_s + \Gamma \sum_{s=0}^{N-1} a_s^2 \quad (21) \]

where \( \Gamma \) is the Lagrange multiplier.

The partial derivatives of \( f \) with respect to the \( a_s \) have to be equal to zero. It results a relationship between \( \Gamma \) and the \( a_s \) that have all to be equal to \( a \). This condition on the \( a_s \) corresponds to the best focus plane of the object. With a simple physical interpretation, we
observe that the extremum of $M_d$ is a maximum. Therefore $M_d$ is maximum when the best focus plane is reached.

3. Experimental results

In the following, we provide results on actual digital holographic data for two pure amplitude and one pure phase objects. For the first amplitude object, the complete digital hologram is processed to compute the focus criterion. For the two other examples, we perform the processing in regions of interest (ROI) around the objects because they are of small size and larger part of hologram could involve other objects that are not in the same focus plane. In order to alleviate disturbances by border effects, the amplitude of the holograms are centered in an larger 1024 x 1024 area where the outside regions are filled with the border amplitude lines of the hologram: The top and bottom outside regions are filled line by line with, respectively, the top and the bottom amplitude lines of the centered hologram while the left and right outside region are filled by the left and right columns.

3.1 Amplitude objects

We present results of this method on digital holograms that were recorded with a digital holographic microscope working with partially coherent illumination [16]. A first test is performed on a digital hologram of a ruler defocused by a distance of approximately 65µm, as show by Fig. 3. The optical phase is obtained by using a 4-Frame phase stepping method and the intensity by recording an image with the reference beam blocked. The microscope lenses have a numerical aperture of 0.15. The field of view is 710 µm X 710 µm recorded on 512 x 512 pixels. The objective is to measure $M_d$ for different refocusing distances. As expected, the minimum of $M_d$ occurs at the refocus plane shown by the Fig. 4 and Fig. 5, where Fig. 5 is a plot of the evolution of $M_d$ as a function of $d$. It confirms that $M_d$ is minimum for the reconstructed plane at the distance $d = -66$ µm from the recorded plane. The performance of the refocusing can be assessed on the image of Fig. 4 where it is observed that the ruler is focused.

Fig. 3. Intensity image of the ruler in the recorded plane.
We consider now a test performed on a digital hologram representing a particle of 5 µm diameter. In this case, the digital holographic microscope uses a partially coherent source created by a laser incident on a rotating ground glass [34] and the detailed operating mode is described in reference [35]. The field of view is 330 µm x 330 µm on a 1024 x 1024 pixels CCD sensor size. The object is not in the plane focused by the imaging system. To obtain the defocus intensity image shown by the Fig. 6, the complex amplitude is computed by the Fourier method on the recorded digital hologram [36]. The reconstructed intensity according to the minimization of $M_d$ is shown by Fig. 7. The evolution of the $M_d$ with respect to the distance of propagation is provided by Fig. 8. Again the minimum occurs when the focus distance is reached.
Fig. 6. Defocus intensity image of 5 µm particle in the recorded plane. ROI of 209 x 201 pixels.

Fig. 7. Reconstructed intensity image of the ROI of Fig. 6 with d=55 µm computed by the focus criterion.
3.2 Phase object

To experimentally demonstrate the method to detect the best focus of pure phase object, we take an example of an in vitro living cell. The objective of this biological application is to analyze cancer cell migration in a 3D matrix gel. They are almost completely transparent and can be considered as phase object. As the cell migration is a slow phenomenon in comparison with the typical image acquisition time, we used a digital holographic microscope working with the phase stepping method [16]. The light source is a 660 nm Epitex LED with a 20 nm spectral width. The microscope objectives are 10x Leica objectives HC PL Fluotar, NA= 0.30. The camera is a Hamamatsu Orca 1 with 1024 X 1024 pixels for a field of view of 685 µm x 685 µm.

Fig. 8. Focus criterion as a function of the reconstruction distance. It is minimal at the distance d=55 µm.
Fig. 9. (a) Intensity image of a cell at a defocusing distance of -30 µm. The ROI size is 111x153 pixels; (b) Phase image corresponding to the image of (a); (c) Intensity image corresponding to (a) reconstructed at a distance d=30 µm. We observe that, in intensity, the object is almost invisible due to the fact that it is a phase object; (d) Phase image corresponding to the image of (c).

Figures 9(a), 9(b), 9(c) and 9(d) show the results that we obtain by using the focus criterion for the phase objects. Figure 9(a) and 9(b) are respectively the intensity and the phase image of a living cell recorded with a defocus distance of -30 µm. By using the distance \( d \) computed thanks to the focus criterion for the phase object, we reconstruct the intensity and phase images of Fig. 9(c) and 9(d). As expected with a pure phase object, it is almost invisible in intensity. However, those phase and intensity information can be used for further phase contrast process.

The evolution of \( M_d \) is plotted in the Fig. 10 where we observe that we obtain a well-shaped maximum when the focus plane is reached.
4. Conclusions

We present a method to determine the refocus distance of object in digital holographic microscopy by transmission that requests few computation times. It is based on a specific focus measure that is equal to the integration of the amplitude modulus. It is shown that this measure is minimized for pure amplitude objects while it is maximized for pure phase ones. The method is demonstrated on actual digital holographic data. The pure phase object refocus criterion is based on the fact that when the image of object is adequately refocused, a constant value of amplitude modulus is reached. It has to be pointed out that in practice, some deviations from this ideal constant amplitude case can be observed [37, 38]. Indeed, when very small phase objects are under investigation in high-resolution configurations, the light spread introduced by the object may be larger than the numerical aperture capability of the imaging system. Therefore, some darkened zones of the reconstructed image can be observed. At this time, the selection of the pure phase or amplitude criteria has to be decided by the user. As in microscopy, the objects can be often considered as belonging to one of these two cases, there are few ambiguities. For example, most of the living cells in liquid can be considered as pure phase objects while opaque particles for velocimetry are pure amplitude objects. However, we have to mention that transparent particles can behave as amplitude objects when they are largely spreading the light. A similar behaviour could be also observed for liquid droplets in air. In those cases, the pure amplitude criterion gives better results. The perspectives are to extent the processing for objects that present this dual amplitude and phase nature and for the determination of the best focus planes of a set of objects that could occur at different locations in the same digital hologram.

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