Study of the Influence of Different Optical Properties on the Image of Compound light microscope

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Abstract:

Microscope images are characterized by a number of specific parameters, the influence of such parameters (intensity, magnification, numerical aperture, diaphragms aperture, segmentation, and edge detecting technique) on measurement in optical microscope images have been determined with using a powerful image processing methods. As one of the most widespread techniques in biological investigation and dynamic process, light compound microscopy has used to analyze the optical properties of biological images. The results indicate that a wide aperture allows maximum resolution and depth of field, but decreases the contrast. While a small aperture improve visibility and contrast but decreases the resolution. The results also show the best performance in focus with image that have a wide diaphragm aperture at high intensity. The analysis of statistical properties of images is acceptable, and there is unnoticeable changes in correlation coefficients between images at high intensity.

Keywords: light Microscopy, Optical Properties, Image Processing.

دراسة تأثير الخصائص البصرية على صور المجهر الضوئي المركب

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كلية التربية، الجامعة المستنصرية، بغداد، العراق

الخلاصة:

تحدد الصور المجهرية بعدد من الخصائص البصرية ، في هذا البحث تم دراسة تأثير الخصائص البصرية التالية (الشدة، عامل الكبير، الفتحة العددية، فتحة الحاجز ، التجزئة، مؤثر كشف الحافة) على صور المجهر البصري المركب. وقد اشارت النتائج ان زيادة فتحة الحاجز تزيد من وضوحيه الصور ومن عمق المجال على حساب التباين. اظهرت النتائج ايضا ان افضل عمق بؤري في الصور الملتقطة كانت عند فتحات حاجز كبيرة وشدات اضاءة عالية .وقد كانت الخصائص الاحصائية مقبولة، اما تغير معامل الارتباط فقد كان غير ملحوظ للصور الملتقطة عند الشدات العالية .

I. Introduction:

Light microscopy is one of the most powerful and widespread techniques that used in the field of cell biology and cytogenetics to be examined as living samples or under conditions that closely approximate the living state [1]. The image information in optical microscopy is based on three fundamental actions: diffraction of light by the specimen, collection of diffracted rays, and interference of diffracted and non diffracted rays in the image plane. The key elements in these microscopes imaging system is the objective lens, which determines the precision with which these actions are diffracted [2]. A critical point in the automation of microscope image acquisition is bringing the specimen into focus before measuring any feature of the images taking [3]. The properties of light allow a small deviation from the focal plane without observable loss of sharpness (depth of field) and this depth of field (DOF) depends on the light collecting power of the objective (numerical

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aperture NA), the wavelength (λ) of the image forming light, and the refractive index of the immersion medium between the specimen and the objective lens [3]. Diffraction which is intrinsic to the physics of lights limits the microscope spatial resolution, especially at high magnification.

In order to collect valid information from images derived from optical microscopy image processing is necessary to extract the maximal amount of information available from the specimen images [4], such processing method applied to these images [5, 6]. The removal of out-off-focus information that contaminates each image requires accurate knowledge of the image forming properties of the microscope.

Investigation, by a variety of researcher are being made in the past years. *Tao et al*. have introduced a confocal fluorescence microscope with adaptive optics, the results show 4.3x improvement in the Strehl ratio and 240% improvement in the intensity for mouse tissues [7]. *Martin et al* they discussed the use of adaptive optics as an effective means to overcome the aberration problem in super resolution microscopy used in the biological investigation [8]. *Cole et al*. have determined the resolution and identify the quality of the microscopes images using confocal microscope [9]. *Levoy et al*. Have demonstrated a prototype light field microscope and show that synthetic focusing followed by 3D deconvolution is equivalent to applying limited – angle tomography to the 4D light field [10].

II. Evaluation of the image quality of optical system:

1- Numerical Aperture:

The numerical aperture is the angle over which the objective can collect diffracted rays from the specimen and can be say it's the key parameter determining spatial resolution [2, 11]. In the optical microscope, the angular aperture is described in terms of the numerical aperture (NA) as:

$$NA = n \sin \theta \qquad \qquad ...1$$

where θ is the half of the cone of specimen light accepted by the objective lens, n refers to the refractive index of the medium between the lens and the specimen. For dray lenses used in air, $n = 1$.

2- Resolution:

For objects illuminated by incoherent light, the Rayleigh resolution limit pertains to two luminous point is [10, 9]:

$$d = 1.22 \frac{\lambda}{2NA} \qquad \qquad ...2$$

1.22 is a geometrical term based on the average 20:20 eye, d: is the minimum resolved distance in μm, and λ is the wave length in μm [2].

This equation describes the Rayleigh criteria for the resolution of two diffraction spots in the image plane with closely distance between them. The optical limit of spatial resolution is important for interpreting microscope images.

Although there are several different theoretical resolution formula, the resolution for the optical microscopy can be calculated on the basis of Rayleigh criterion. Many microscopists select other resolution criteria, but all of these choices are only mathematical approximation of the same physical properties. There are two major methods used to determine the resolution of an objective lens and, in effect of the microscope itself [2, 12, 13], the resolution in one direction (axial resolution) which is defined as the ability to distinguish features at different depths by refocusing the microscope. The commonly accepted measure of axial resolution is depth of field (DOF) [13]:

$$DOF = \frac{\lambda n}{NA^2} \qquad \qquad ...3$$

Where n is the refractive index of the medium between the lens and the object, λ is the wave length of light in air [2]. The resolution in two direction is the (lateral resolution) [14, 15]. A commonly used measure of this resolution is Rayleigh limit which agrees well with experimental values, Expressed as a distance on the intermediate image plan, this limit is:

$$R = \frac{\lambda}{2NA} \qquad \qquad ...4$$

3. Depth of field and depth of focus:

The image of a point object is a diffraction disk of finite diameter, so the disk that determines using the laws of diffraction has a measurable thickness along the z-axis. The thickness of the optical section along z-axis within which objects in specimen are in focus defined as the depth of field DOF (z) in the object plane; the thickness of the image itself is the depth of focus [13, 2].

The purpose of the extended depth of focus algorithms is to recover from each slice of images the pixel that is in focus [3, 6]. The function we use to compute extended depth of focus is the variance [16]. This function applied to the images in order to select the image that closed to the focal plane of the objective. The variance function is [6]:

$$V(I) = \sum_{i=0}^{N} \sum_{j=0}^{M} [I(i,j) - \mu]^2 \qquad \qquad ...5$$

μ is the mean gray level given by:

$$\mu = \frac{1}{NM} \sum_{i=0}^{N} \sum_{j=0}^{M} I(i,j) \qquad \qquad ... 6$$

The slice with the highest variance was selected as a reference image [16].

Another method was used to compute the extended depth of focus is Sobel operator. The Soble operator [17, 3] is employed to select the best in-focus slice. The Soble operator is an edge detector returns a measure of the strength of an edge being present at a given pixel. Distinctly in-focus regions will have strong edge present. The Soble operator which represents a magnitude is given as:

$$M = \sqrt{G_x^2(i,j) + G_y^2(i,j)} \qquad \qquad ... 7$$

$$G_x = \begin{bmatrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{bmatrix}, \quad G_y = \begin{bmatrix} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{bmatrix}$$

4. Contrast:

Contrast produced by the absorption of light, brightness or color at different points in the specimen and defined as the difference in the intensity of light between the specimen and the adjacent background relative to the overall background intensity. In term of simple formula [16, 13]:

$$C = \frac{I_{max} - I_{min}}{I_{max} + I_{min}} \qquad \qquad ... 8$$

From this equation is evident that the specimen contrast refers to the relationship between the highest and lowest intensity in the image. I_{min}, and I_{max} are intensities measured from the specimen and background [16, 18].

Contrast dependent upon interaction of the specimen with light, so is not an inherent property of the specimen [19]. Control of images contrast in the microscope optical system is dependent upon several factors including intensity and wavelength of light, magnification factor, and proper setting aperture diaphragms.

III. Optical Properties of Human Tissue:

There is an interest in study the optical properties of human tissue in order to find the best way of detecting and locating lesions and tumors. The image perceived with a microscope is formed by light that interacts with the specimen or tissue placed on the stage. Besides the direct effects of the wave nature of light (diffraction phenomena), other effects based on the wave or particle nature of light are responsible for absorption and scattering in tissue. Most of the absorption of light is by the various pigments and enzyme's in the cell, by water and by body fats. And most light scattering in tissue is believed to take place at the various membrane boundaries, both the cell membrane as well as the membrane of the various organelles inside the cell [2, 3].

IV. Optical System of Microscope:

The optical system of microscope differ from those of camera. In a microscope seen, light field capture and display can be analyzed using a geometrical optics, and in a microscope seen, most objects scatter light making them opaque [10].

The optical properties of a microscope system are dominated by the behavior of the objective lens, which is the arguably the most important component of any light microscope [4]. A compound light microscope is an optical instrument that use visible Light to produce a magnified image of an object (or specimen) that is projected on to the retina of the eye or on to an imaging device [16]. The objective lens and the eyepiece (or ocular), work together to produce the final magnification M of the image such that [2]:

$$M_{final} = M_{obj.} + M_{oc.} \qquad \qquad ...9$$

The objective lens collects light diffracted by the specimen and forms magnified real image at the real intermediate image plane near the eyepieces, the condenser lens focuses light from the illuminator on to a small area of the specimen.

Figure-1 [2] shows how an image becomes magnified and is perceived by the eye, the Figure also points out the location of important focal planes in relation to the objective lens, the ocular, and the eye. The light microscopy has an ability to allow biological conditions that closely approximate the living state [4]. The magnification M, which in the illustration is 100x and its written in the largest font. The NA is written next to the magnification, but in smaller font. Below the magnification and numerical permute, the tube length (∞) and the cover slip thickness are given.
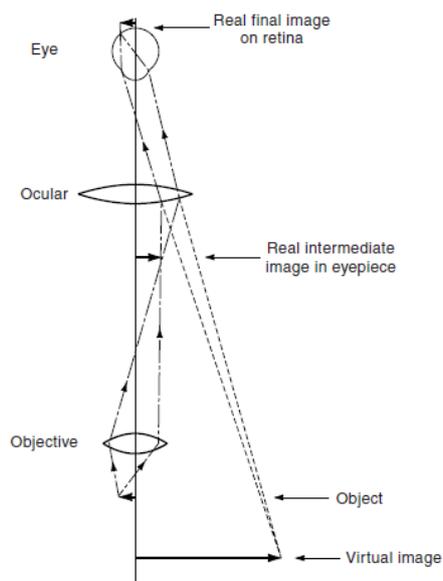
Figure 1- Perception of a magnified virtual image of a specimen in the microscope [2]

When light from microscope lamp passes through the condenser and then through the specimen, some of light passes both around and through the specimen undisturbed in its path, the background light (surround light) passing around the specimen is also undeviated light. Some of light passing through the specimen is deviated when it encounters parts of the specimen. Such deviated light (diffracted light) is rendered one-half wave length or 180 degrees out of phase with the direct light that has passed through undeviated. The one-half wave length out of phase, caused by the specimen itself, enables this light to cause destructive interference with the direct light when both arrive at the intermediate image plane located at the fixed diaphragm of the eyepiece [18] then this image further magnificence by the eyepiece. Which finally is projected on to the CCD camera [4, 20].

V. Correlation:

Correlation is a method for establishing the degree of probability that a linear relationship exists between two measured quantities the correlation coefficient has the value $r = 1$ if the two images are absolutely identical, $r = 0$ if they are completely uncorrelated and $r = -1$ if they are completely anti-correlated. The correlation coefficient is defined as: [21, 22]

$$r = \frac{\sum_i (x_{i-x_m})(y_{i-y_m})}{\sqrt{\sum_i (x_{i-x_m})^2} \sqrt{\sum_i (y_{i-y_m})^2}} \qquad \text{... 10}$$

Where, x_i and y_i are the intensity values of ith pixel in 1st and 2nd image respectively. And, x_m and y_m are the mean intensity values of 1st and 2nd image respectively.

VI. Results:

The 2D image forming properties were experimentally examined using an optical compound microscope. The light source was a lamp which transmitte incoherent light (400-700 nm) to the microscope; all images were acquired with CCD camera. The images were digitized in a format of 512*512 pixels and stored in the JPEG format. Four different setup of images compared to study effects caused by different intensity (i), diaphragm aperture (g), and magnification (M). While there

are similarities there are significant difference between these sets of images. Figure-2 shows images obtained for 40X and 10X objective, the images on the lower low were acquired using green light (546 nm). Figures-3a, b shows images through different diaphragms aperture sizes (g), and Figure-4 shows images obtained using different illumination intensity (i).
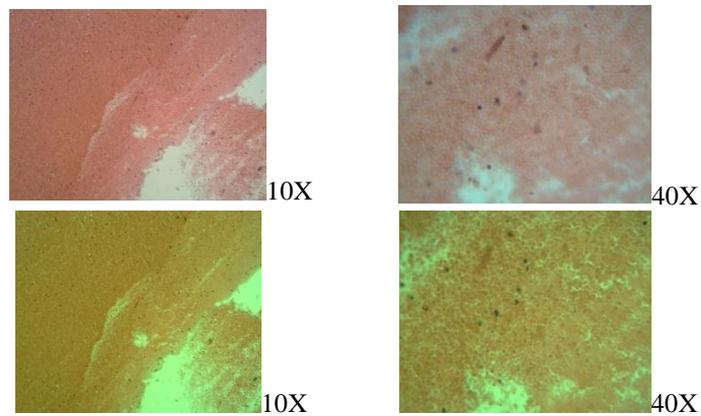
Figure 2- Different magnification, upper: images acquired using normal light.
Lower: images acquired using green light

i=100, g=20   i=100, g=50   i=100, g=75   i=100, g=100

i=50, g=100   i=50, g=75   i=50, g=50   i=50, g=20

a)

i=25. g=100   i=25, g=75   i=25, g=50   i=25,g=20

b)

i=100, g=20   i=100, g=50   i=100,g=75   i=100, g=100
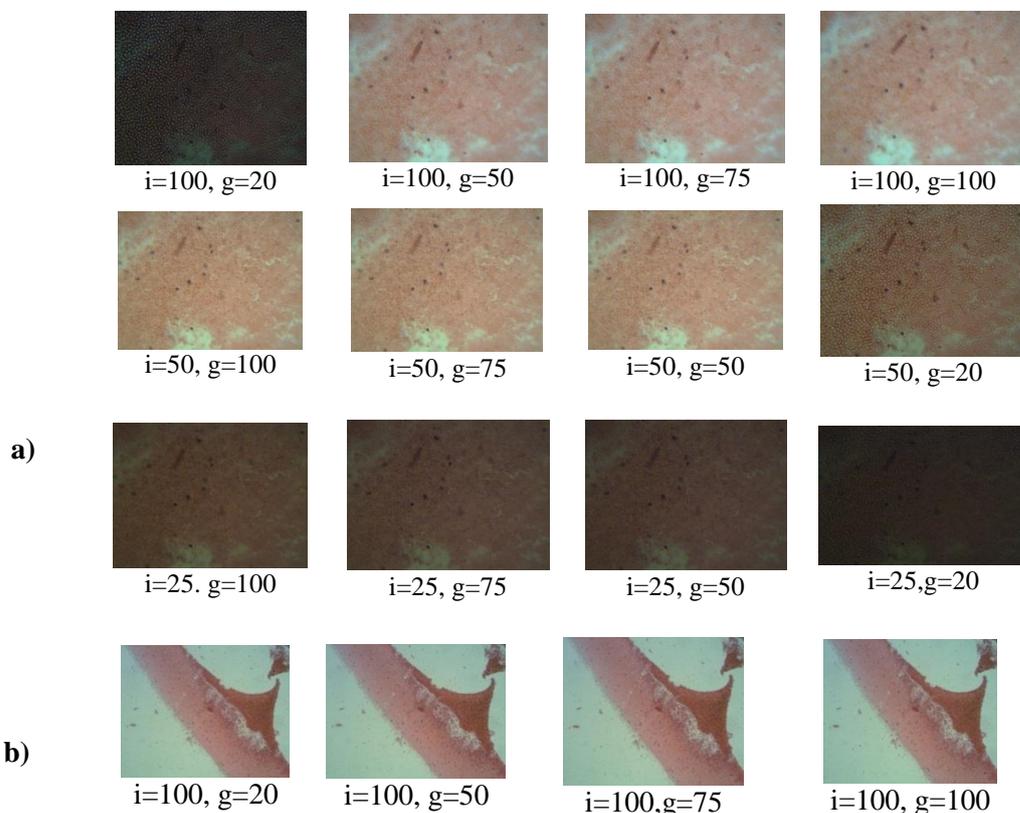
Figure 3- a) images through different aperture diaphragms (40X)
b) images through different aperture diaphragms (10X)

g=100, i=100   g=100, i=75   g=100, i=50   g=100, i=25
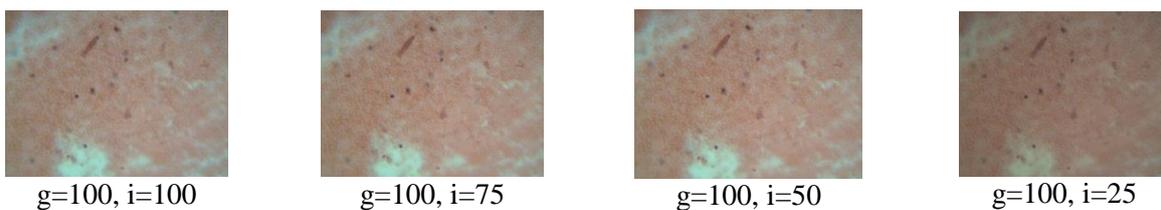
Figure 4-Images obtained for different illumination (40X)

1. Influence on Contrast:

The experimental results were carried out for contrast are illustrated in Figures-5,6, the graph presented in Figure-5 shows the effect of intensity on the contrast of specimen image, images show a weak contrast with decreases the intensity of light ,as the intensity increase the borders of the individual squames are Cleary shown that means high contrast. As shown in Figure-6 a, b the contrast decrease with a wide aperture, because a wide aperture decreases contrast .while a smaller constricted aperture improve visibility and contrast, the contrast is small for 40X objective. Table-1 shows a weak contrast when the specimen illuminated with green light and the contrast is small for 40X objective.
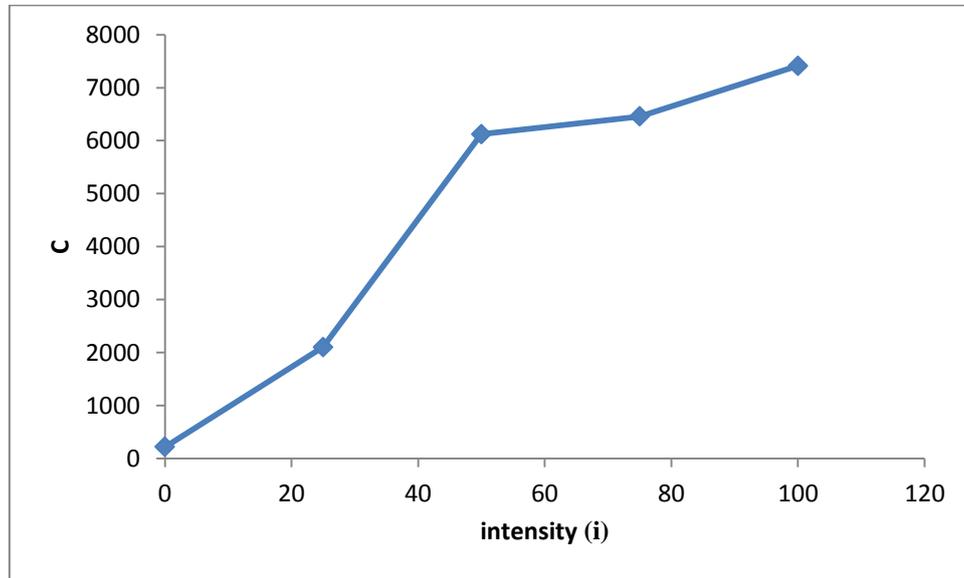
Figure 5- effect of intensity on the contrast
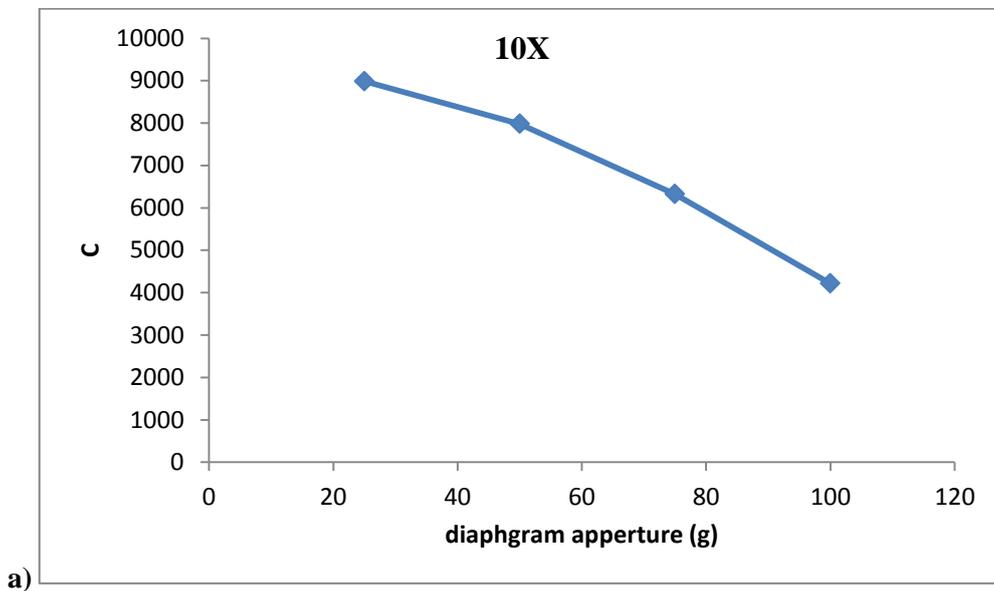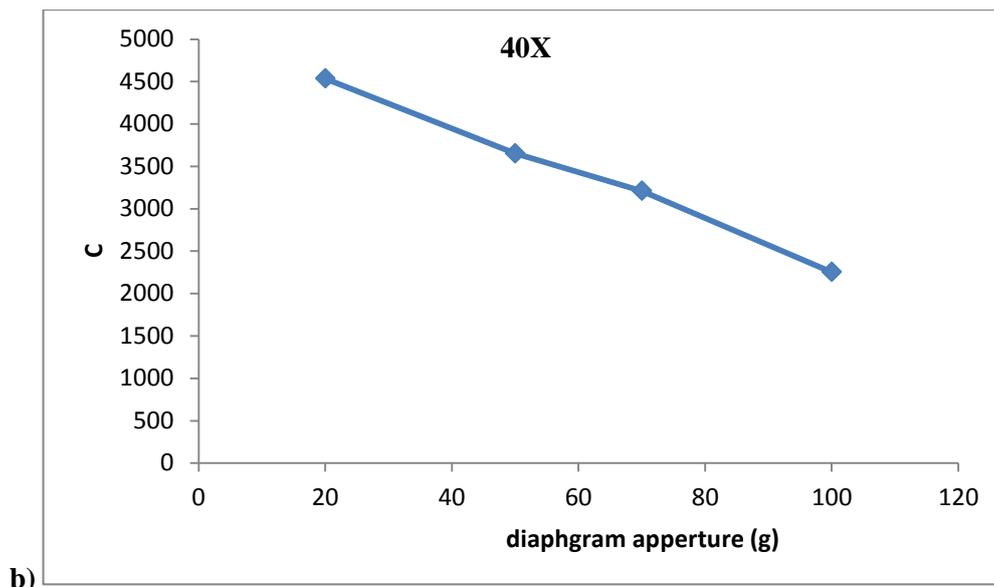
Figure 6 - a, b effect of the aperture on the contrast

Table 1- The contrast of images illuminated with white and green light

Image				
	10X	10X (green)	40X	40X (green)
Contrast	9478.11	8574.45	7532.51	6325.79

2. Influence on Resolution:

When observing the image through the eyepieces it is much easier to get a sharp image than when observing the specimen with a camera. Unlike the human eye, a camera lens cannot accommodate, which makes it more difficult to focus the specimen in automated imaging.. The theoretical resolution (lateral resolution, axial resolution) of the optical microscope under the condition (green light, white light with 40 X objectives) was calculated using equations (3, 4) as shown in Table-2.

Table 2- Results of Lateral and axial resolution

	10/0.25NA	40/0.65 NA
R_g	1070 nm	411.538 nm
R_y	1200 nm	461 nm
$(DOF)_g$	8560 nm	1266.2 nm
$(DOF)_y$	9600 nm	1420.11 nm

It is obviously from the numerical results in the Table-2 that the higher the NA, the shallower will be the DOF. Experimental result was provides directly from the Figure-6, a wide aperture decreases the contrast, but allows maximum resolution and that will increase DOF. The shorter the wavelength of visible light used, the better the resolution.

3. Compute the Extended Depth of Focus on Images:

The variance function was applied to the set of images listed in Figure-3a at different diaphragm aperture (g) with different intensity (i) in order to select the image that closed to the focal plane of the objective, the whole (512*512) image was used with the variance measure. Figure-7 shows the plot of variance values for the images set, the slice with the highest variance was selected as a reference image, where the maximum corresponds to the image at high illumination and unrestricted aperture.
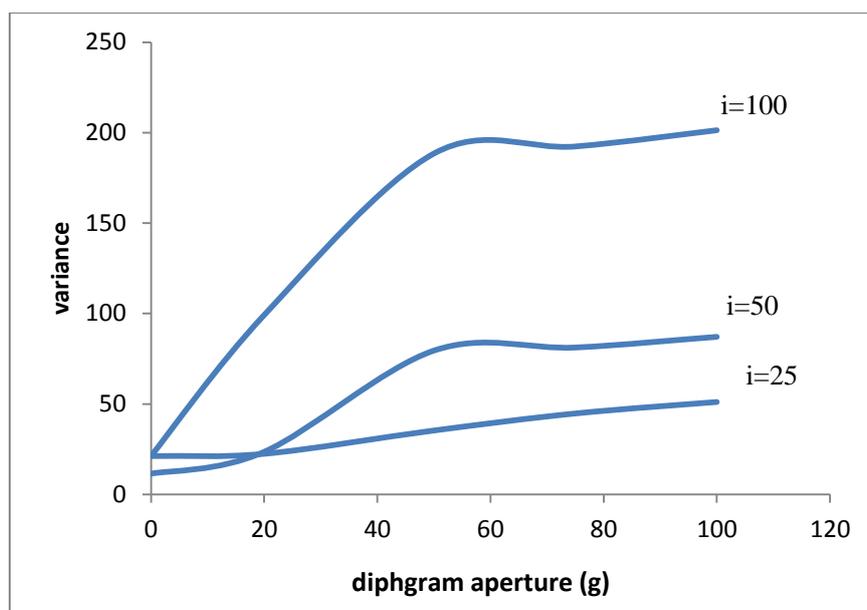
Figure 7- The variance for the set of images

Figure-8 displays some of the results after applying Sobel operator on the same original images displayed in Figure -3a.

The main steps in edge detection are:

- First derivative: Sobel operators.

1. Smooth in one direction, differentiate in the other.
2. Apply Sobel mask for x-direction.
3. Apply Sobel mask for y-direction.
4. Found the absolutes value.
5. Found the arctan= gradient direction.
6. Found the gradient of the image.
7. Define a threshold value.

The edge detection techniques were implemented using MATLAB. That is a great and easy tool to use to simulate image process.

| **a- g=20** | **b- g=50** | **c- g=70** | **d- g=100** |
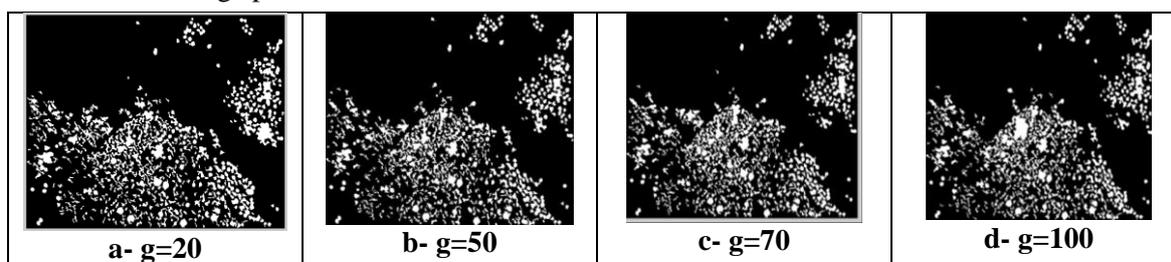
Figure 8- Some of the images after applying Sobel operator with i=100

The best edge found is in the image that means the best performance in-focus with image that have a wide diaphragm aperture at full illumination.

4. Statistical Study:

To get more information on the influence of optical parameters, Table-3 shows several statistical measurement of images captured under high illumination conditions (i=100) for different diaphragms Figure-9. From the table, the area and diameter measurement results indicate that the size increase with increasing aperture. We find that the best intensity of pixels in case when image is captured under high illumination conditions. The results of mean and standard deviation summarized in Figure-10a, b.

| i=100, g=0 | i=100, g=75 | i=100, g=50 | i=100, g=20 | i=100, g=100 |
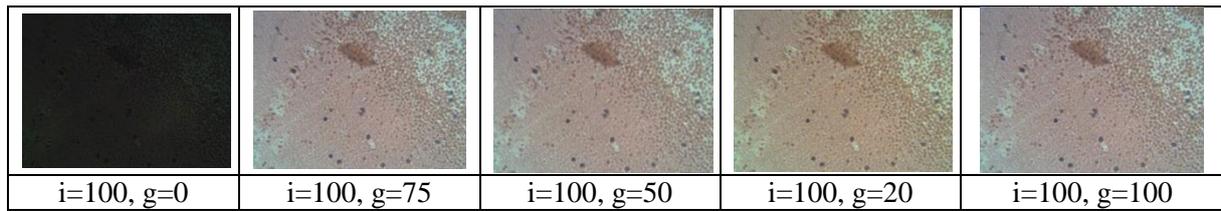
Figure 9- Images through different aperture diaphragms (i=100).

Table 3- Several statistical measurement of images in figure (8)

Samples	Image1 i=100,g=0	Image2 i=100, g=20	Image3 i=100,g=50	Image4 i=100, g=75	Image5 i=100, g=100
Mean	133.2525	145.2697	143.4241	142.1244	142.0973
Intensity	1360.722	1313.171	1315.643	1412.915	1391.355
Area	188.1001	224.2934	229.5069	237.7398	233.1697
Perimeter	2086.949	1181.254	1200.698	1197.125	1204.666
Centroid	726.9486	1123.751	1133.107	1145.522	1139.816
Diameter	37.19097	35.54494	35.7181	36.07826	35.92505
roundness	0.598611	0.447472	0.441221	0.436957	0.439476
redMean	142.6344	154.6449	152.7672	151.5939	151.5337
greenMean	148.3849	145.1821	145.3177	145.5271	145.5725
blueMean	145.1879	126.6359	139.4473	142.2034	142.475
skewness	0.00625	-0.01055	-0.0385	-0.03773	-0.03186
kurtosis	2.130139	2.168004	2.151502	2.172947	2.164095
Energy	0.002909	0.003216	0.003101	0.00308	0.003162
Homogeneity	0.009239	0.011126	0.010888	0.010784	0.010986
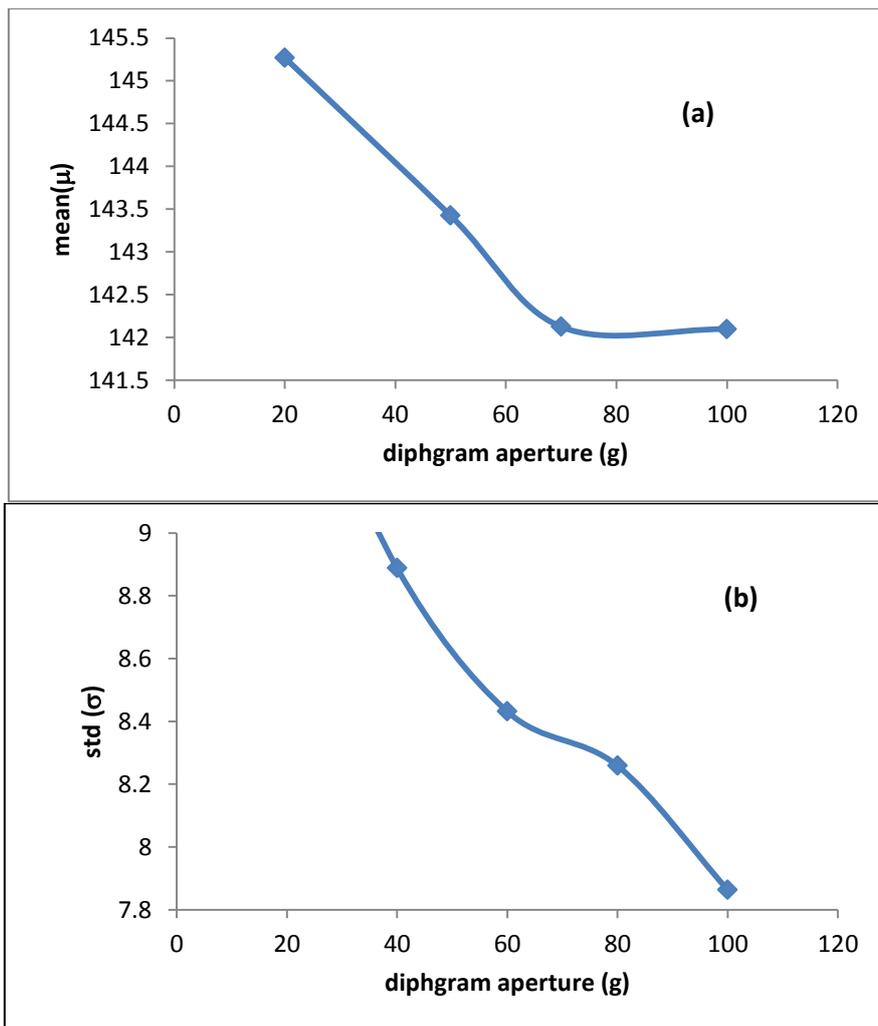
Figure 10- a, b Results of mean and standard deviation

The training data set used in the experiments to compute the correlation consists of the images from Figure-9 and the Pre-processing of these images includes following steps:
- Resize images to size of 120x120.
- Conversion of images from RGB color space to gray scale.
- Integer to double precision conversion.

From Table-4, it is concluded that correlation coefficient has high overall recognition rate between the images that captured under high illuminated conditions.

Table 4- Result of correlation function

Image intensity	Correlation coefficient
g=75,i=100	0.8218
g=75,i=100	0.9919
g=75,i=100	0.9926
g=75,i=100	0.9932

VII. Discussion and Conclusion

With the experimental results and theoretical analysis presented in this paper, we can conclude that: a wide aperture allows maximum spatial resolution, but decreases the contrast, while a smaller constricted aperture improves visibility and contrast, but the spatial resolution decreases. As we have mentioned the wavelength of light is an important factor in the resolution of a microscope this is obvious from the result   that shows the shorter the wavelength of visible light used (green light) yield higher resolution. And images show weak contrast when illuminated with white light and the higher the NA, the shallower will be the DOF. Also We noted that the excellent image extended focal depth in light microscopy is at high illumination and unrestricted aperture and infocus area in an image clearly give rise to sharp edges in an image. The results of statistical investigation of the set of images give good results, and the best correlation between images captured is under highly illuminated conditions.

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