PROBLEM SET 1
Ptandon1

Question #01 (10 points) Phase contrast and DIC are two commonly used contrast generation techniques. Briefly explain their similarities and differences.

Ans 1) The major differences between the phase contrast technique and differential interference contrast technique are as follows:

- *Contrast formation mechanisms*

  The image intensity produced in phase contrast technique varies with the specimen's optical path length. This is not so in the case of DIC where optical path length gradients are primarily responsible for contrast.

- *Phase contrast has halo formation.*

  The phase contrast images tend to have a bright region around the edges of the objects under focus called as 'halo' effect. This adds noise to the normal pixel intensities and makes it hard to detect edges by gradient analysis. These halos are absent in the case of DIC.

- *Depth information with phase contrast*

  Phase contrast is not able to distinguish if structures are present outside cell membrane or lying on the underside of the cell.

- *DIC can use the instrument at the full NA*

  This property improves the axial resolution to produce high resolution images at high NA and is due to the fact that DIC omits the masking effects of phase plates or condenser annuli.

- *Sample Opacity and staining*

  A limitation to phase contrast is due to the fact that it is mainly used in case of images having poor contrast category while for DIC, stained and unstained samples are all equal when it comes to usage but the only drawback being that it suffers from birefringement features.

The similarities between the techniques include:

Both are contrast enhancing techniques used in the field of bioimage informatics. Both are capable of producing high contrast images of transparent biological phases that do not ordinarily affect the amplitude of visible light waves passing though the specimen. Both have a phase shift detection limit of $< \frac{\lambda}{100}$.

Question #02 (10 points) Excitation spectrum and emission spectrum provide critically important information about a fluorophore. Briefly explain why they are often horizontally flipped mirror images of each other.

Ans 02) Spectrum, (be it excitation or emission) is mainly a result of the interactions between photons and atoms. When an atom absorbs photons and gets into the excited state. The spectrum of light that is capable of switching the atoms from their ground state to their excited state is known as excitation spectrum. On the other hand, when such an excited atom returns from the excited state to the ground state, it emits photons. These photons form a spectrum of light. In the ideal conditions both the excitation and the emission spectrum must be the same. But due to a phenomenon known as Stoke's

shift the emission spectrum wavelength is shifted more towards longer wavelengths and the peak of the emission spectrum is lower than that of the excitation spectrum. When both the spectrum are plotted, they are found to be mirror images of each other. The higher the Stoke's shift, the easier it is to separate the excitation and emission spectrum and vice versa. One main application of this phenomenon is the identification of the photons isolated from the waves by the sample under study.

Question #03 (10 points) Briefly explain what determines the resolution of a fluorescence microscope. What is the Rayleigh limit? How is it different from the Sparrow limit?

Ans 3) The resolution of a microscope is the smallest distance at which if two points are placed, can be identified as distinct objects when viewed through the microscope. In case of a fluorescence microscope, this is attributed to the distance between two illuminated fluorophores.

Rayleigh limit corresponds to the case when the principal maxima of one of the points overlaps with the first diffraction minima of another fluorophore. If the distance between two fluorophores is greater than this distance, they are said to be resolved otherwise they sort of merge together and are not resolvable. This can also be interpreted in other words as: if the objects/points are situated at a distance larger than the PSF, they are resolved otherwise not.

The Rayleigh limit is given by the following equation:

$$Rayleigh\ Resolution_{x,y} = \frac{0.61\lambda}{NA}$$

The sparrow limit on the other hand is the minimum distance between two point sources such that constant brightness is shown across the region between the peaks. It is approximately given as 2/3rd of the Rayleigh Limit.

The Sparrow limit is given by the following equation:

$$Sparrow\ Resolution_{x,y} = \frac{0.47\lambda}{NA}$$

Question #04 (10 points) Photobleaching and phototoxicity are two commonly encountered problems in fluorescence microscopy. Briefly explain what they are, where they come from, and how they will impact image collection.

Ans 4) Photobleaching is: the reduction in the ability of a fluorophore to emit photons and generate fluorescence due to prolonged exposure to light.

- Origin: The effect is caused due to covalent modification and photon induced chemical damage. It is possible that after the excitation due to the incident light, the molecules may interact and reach an irreversible stable covalent stage such that they do not go back to their initial low energy levels. This leads to lower number of photons emitted as a part of fluorescence.
- Impact on Image collection: The image intensity and thereby the image quality is reduced and is sustained. Reducing the light intensity in this case does not actually eliminate the photobleaching from happening, it merely reduces the rate.

Phototoxicity is: the destruction of the sample under observation due to exposure to excessive amounts of light.

- Origin: Too much exposure to light generates free radicals inside the cells under observation. Free radicals are chemically active compounds that have a single electron in their outermost shell. These compounds cause damage similar to oxidative damage or oxidation of the sample under study.
- Impact on image collection: While there is not much difference in the beginning, if the sample is exposed to longer periods of time, the image cannot be taken any further due to sample destruction. This places a limit on the how many frames of images can be collected. Thus such samples are exposed to very brief and well gapped periods of light and taken their image. This ensures that the cells do not get oxidized and images can be taken again and again.

Question #05 (10 points) Briefly describe different types of noise in a fluorescence microscopy image.

Ans 5) Like every other possible means of scientific measurement, fluorescence microscopy suffers from the errors associated in the measurement. This source of error in case of flurescence microscopy is due to the noise. Noise is used as a generic term that hampers our ability to perform microscopy in the perfect sense and also reduces the efficiency to analyze the data generated because it hides the real data among the random values present in the image.

The various types of noise associated with fluorescence microscopy are as follows:

- Signal Shot Noise(N_{shot}): This is related to the error associated with the distribution of photons which is considered to be a Poisson distribution. It is calculated as follows:

  $N_{shot} = \sqrt{S}$ where S is the signal (electron) given by $S = I.QE.T$ (QE -> Quantum efficiency)

- Camera Noise(N_{camera}): The camera noise is actually a combination of two kinds of noise associated with the camera. The first one is known as the read noise and is associated with the part when the image is read by a detector and converted into a voltage signal. (N_{read}).

  The second type of noise associated with the camera is the dark noise N_{dark}. The environmental heat around the detector mainly contributes to the production of dark current and the fluctuation of which contributes to the production of dark noise. Thus,

  $$N_{dark} = \sqrt{D.T}$$

  Where D is the dark current and T is the time.

  And so $N_{camera} = \sqrt{N_{dark}^2 + N_{read}^2}$

  The total noise of the system is contributed by Camera noise and the Read Noise and is equal to:

  $$N = \sqrt{N_{camera}^2 + N_{shot}^2}$$

The camera noise stays the same and does not depend on the light intensity. The shot noise on the other hand is totally dependent on the light intensity. Shot noise actually dominates the total noise at higher light intensity levels.

Question #06 (10 points) Briefly explain why a 2D Gaussian kernel is separable. Briefly explain how this property can be used to reduce computational cost in image filtering for noise suppression as well

as for calculating first-order and second-order image derivatives. Please be sure to include key equations in your answer.

2D Gaussian kernels are separable because of their unique property that they can be represented as a product of individual 1D kernels.

Example: The equation for a 2D Gaussian Kernel is given by:

$$N(x, y, \sigma_x, \sigma_y) = \frac{1}{2\pi\sigma_x\sigma_y} e^{-\left(\frac{x}{2\sigma_x^2}+\frac{y^2}{2\sigma_y^2}\right)}$$

$$= C * e^{-\left(\frac{x}{2\sigma_x^2}\right)} * e^{-\left(\frac{y^2}{2\sigma_y^2}\right)}$$

$$= c_1 e^{-\left(\frac{x}{2\sigma_x^2}\right)} * c_2 e^{-\left(\frac{y^2}{2\sigma_y^2}\right)}$$

$$= N_1(x, \sigma_x) * N_2(y, \sigma_y)$$

This property finds applications in Noise suppression where a Gaussian filter is applied over the image and in the calculations of first and second order derivatives. Since the Gaussian kernel is easily separable, when it comes to calculation of first and second order partial derivatives and noise suppression (by convolution), they can be computed very easily by isolating the Gaussian pertaining to the non-related dimension and applying the derivative operation only on the Gaussian of that dimension. This means:

$$\frac{\partial N(x, y, \sigma_x\sigma_y)}{\partial x} = N_2(y, \sigma_y) * \frac{\partial N_1(x, \sigma_x)}{\partial x}$$

And

$$\frac{\partial N(x, y, \sigma_x\sigma_y)}{\partial y} = N_1(x, \sigma_x) * \frac{\partial N_2(y, \sigma_y)}{\partial y}$$

The process is similar when it comes to the second order derivatives. Since these operations are independent of each other they can be assigned to idle processors in a machine for faster computation of the result that utilizes these derivatives.

Question #07 (10 points) Briefly outline the key steps of a Canny edge detector. Please be sure to include key equations in your answer.

Ans 7) The key steps of a Canny edge detector include:

- *Noise reduction*
  - This involves convolving the image with a Gaussian kernel.
  - If $I[i,j]$ is the image, $G[i,j;\sigma]$, the Gaussian kernel, the resulting convolved image is given by
    - $S[i,j] = G[i,j;\sigma] * I[i,j]$
    - $The\ \sigma\ here\ denotes\ the\ width\ of\ the\ Gaussian\ kernel.$
- *Image Gradient*

- The Canny edge detector searches horizontal, vertical and both diagonal directions for the presence of the edge. This involves computing the gradient in horizontal and vertical direction and then from it computing the direction of the edge.
- We obtain the horizontal(x) and vertical(y) derivatives by the first order finite difference scheme as:
  - $I'_x = \frac{S[i,j+1]-S[i,j]+S[i+1,j+1]-S[i+1,j]}{2}$ and $I'_y = \frac{S[i,j]-S[i+1,j]+S[i,j+1]-S[i+1,j+1]}{2}$
- The edge strength is calculated as:
  - $I'(x_0, y_0) = \sqrt{I'^2_x(x_0, y_0) + I'^2_y(x_0, y_0)}$
- The edge direction is calculated as
  - $\theta[i,j] = \arctan\left(I'_x[i,j], I'_y[i,j]\right)$

➢ *Edge thinning by non-maximum suppression*

- Now that the edge direction and gradient is identified, this step removes those values along the gradient line that are not peaks.
  - First we convert the θ obtained above to corresponding sector angle.
    - $\mathrm{E}[i,j] = Sector(\theta[i,j])$
  - Then we suppress the ridges using $E\ and\ I'(x_0, y_0)$
    - $N[i,j] = nms(I'(x_0, y_0), \mathrm{E}[i,j])$

➢ *Edge tracing through image and hysteresis thresholding*

- Even after the process of NMS, there might be false edge fragments remaining in the matrix N.
- This step aims to reduce the number of those false edge fragments by double thresholding. First a high threshold is set which contains negligible false edges but gaps in contours. Secondly, a low threshold is set that is capable of filling those contours but also having more false edges. A combination of these two images by edge linking is the result of the Canny edge detection algorithm.

Question #08 (10 points) Briefly explain the basic principle of STORM super-resolution imaging. What determines the uncertainty in particle detection in STORM?

Ans 08) STORM or Stochastic Optical Reconstruction Microscopy is based on the unique property of the newly discovered photoswitchable fluorophores that are easily switchable between the ground state and excited state manually. To achieve super resolution, the main challenge that exists is resolution offered by the technique. While other techniques aiming to provide super resolution tend to focus on narrowing the diffraction induced by the background, STORM uses a much more subtle approach.

In STORM, we illuminate a particular set of fluorophores that such that no two illuminated fluorophores are situated at a distance less than the resolving power of the microscope. When imaging of this set of fluorophores is done, they are turned off and another set of fluorophores is illuminated. The process is repeated again until all the fluorophores are covered. As a result of this process, a wide array of images are produced which are then superimposed to get the real image.

Question #09 (10 points) Briefly explain the basic principle of Hough transform for straight line detection. Briefly explain how a Hough transform can be generalized for detection of parametric curves such as circles and ellipses.

PROBLEM SET 1
Ptandon1

Hough transform is based on a voting procedure and it aims to find imperfect instances of certain class of objects in an image/series of images by detection of peaks instead of recognition of a pattern.

Considering a simple example of fitting a set of line segments through a series of points in the image, if two lines are given as:

$y_1 = a.x_1 + b$ and $y_2 = a.x_2 + b$ then transformed versions, $y_1 = \bar{a}.x_1 + \bar{b}$ and $y_2 = \bar{a}.x_2 + \bar{b}$ must pass through a and b in the transformed space.

This kind of parameterization works for all cases except vertical lines. For that purpose we use a different parametric measure.

$$\rho = y \sin\theta + x \cos\theta$$

The curves to be detected can be of arbitrary form as long as they can be parameterized. Hough transform can be generalized to detect circles, ellipses, or any other curve that can be parameterized.

Hough transform for Circles with known radius but unknown center

$$(x - x_c)^2 + (y - y_c)^2 = R^2 \;\rightarrow\; (x_i - x_c)^2 + (y_i - y_c)^2 = R^2$$

Hough transform for Ellipses with known major and minor semi-axes but unknown center

$$\frac{(x - x_c)^2}{a^2} + \frac{(y - y_c)^2}{b^2} = 1 \;\rightarrow\; \frac{(x_i - x_c)^2}{a^2} + \frac{(y_i - y_c)^2}{b^2} = 1$$

Question #10 (10 points) High-throughput/high-content screening is an important area of application for bioimage informatics. Please briefly explain, in your opinion, what the key technological challenges for bioimage informatics are in this area, and how we can overcome these challenges.

Ans 10) With respect to the areas of high throughput screening, bioimage informatics though integral, faces a multitude of challenges for efficient mining of information. With details going upto the scale of nanometers in such images, the computational cost of retrieving feature information from such images gets very expensive even on the scale of a single image. With the addition of noise, the process becomes much more cumbersome.

On the other side, i.e. after the generation of data, the quality control and the validation steps involving real experimental data can be very costly and may not even be representative of the problem at hand.

Regarding the implementations, source codes for developing test protocols are available but may be in some other language/platforms. Apart from these, the most challenging part comes to the tweaking of the parameters to obtain the desired results.