

UNIVERSITY OF KWAZULU-NATAL (HOWARD
COLLEGE)

MASTERS THESIS

Discrete Energy Minimisation Optimisation using Graph Cuts for Fluorescence Microscopy

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*A thesis submitted in fulfillment of the requirements
for the degree of Master of Science in Engineering
in the*

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School of Engineering

September 12, 2016

Declaration of Authorship

I, Ryan NAIDOO, declare that this thesis titled, “Discrete Energy Minimisation Optimisation using Graph Cuts for Fluorescence Microscopy” and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed:

Date:

"Thanks to my solid academic training, today I can write hundreds of words on virtually any topic without possessing a shred of information, which is how I got a good job in journalism."

Dave Barry

UNIVERSITY OF KWAZULU-NATAL (HOWARD COLLEGE)

Abstract

Faculty of Engineering
School of Engineering

Master of Science in Engineering

**Discrete Energy Minimisation Optimisation using Graph Cuts for Fluorescence
Microscopy**

by Ryan NAIDOO

The Thesis Abstract is written here (and usually kept to just this page). The page is kept centered vertically so can expand into the blank space above the title too...

Acknowledgements

The acknowledgments and the people to thank go here, don't forget to include your project advisor. . .

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List of Abbreviations

ACWE	Active Contours Without Edges
AOD	Average Optical Density
BCC	Boundary Chain Code
CED	Coherence Enhancing Diffusion
CCD	Charge-Coupled Device
CLSM	Confocal Laser Scanning Microscopy
CRF	Conditional Random Field
DCC	Differential Chain Code
DNA	Deoxyribonucleic Acid
DT	Delaunay Triangulation
EGFP	Enhanced Green Fluorescent Protein
EM	Expectation Maximisation
FCS	Fluorescence Correlation Spectroscopy
FIFO	First-In First-Out
FISH	Fluorescence in-situ Hybridisation
FLIM	Fluorescence Lifetime Imaging Microscopy
FRAP	Fluorescence Recovery After Photobleaching
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
GLCM	Gray Level Co-occurrence Matrix
GMM	Gaussian Mixture Modelling
ICC	Immunocytochemistry
ICF	Immunocytofluorescence
IHC	Immunohistochemistry
IHF	Immunohistofluorescence
IOD	Integrated Optical Density
Laser	Light Amplification by Stimulated Emission of Radiation
LED	Light Emitting Diode
MAP	Maximum A Posteriori
MIS	Medical Image Segmentation
MLP	Multi-Layered Perceptron
MRF	Markov Random Field
MST	Minimum Spanning Tree
NA	Numerical Aperture
ORI	Optimised Rotational Invariance
PSF	Point Spread Function
RNA	Ribonucleic Acid
SNR	Signal-to-Noise Ratio
TV	Total Variation
UV	Ultraviolet

Physical Constants

$$\begin{array}{ll} \text{Speed of Light} & c = 2.997\,924\,58 \times 10^8 \text{ m s}^{-1} \text{ (exact)} \\ \text{Planck's Constant} & h = 6.626\,070\,04 \times 10^{-34} \text{ m}^2 \text{ kg s}^{-1} \text{ (exact)} \end{array}$$

List of Symbols

a	distance	m
P	power	W (J s^{-1})
ω	angular frequency	rad

For/Dedicated to/To my...

Chapter 1

Introduction

1.1 Outline and Contributions

The introduction is here.

1.2 Thesis Overview

The remainder of the thesis outline.

Chapter 2 is where we cover the mathematical foundation to Graph Cut image segmentation.

Chapter ?? is where we cover the mathematical foundation to Graph Cut image segmentation.

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Chapter 3 is where we cover the mathematical foundation to Graph Cut image segmentation.

Chapter ?? concludes the thesis with suggestions for further work.

Chapter 2

Fluorescence Microscopy

[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13]

What is fluorescence microscopy? What's its purpose in the thesis? Photoluminescence -> fluorescence and phosphorescence. Discovery of fluorescence: Brief history and evolution. Brief discussion on the remainder of the chapter. Fluorescence microscopy has become an essential tool in diverse fields, such as petrology, semiconductors, etc, and has especially been established as a choice imaging technique in cellular and molecular biology for visualisation of cells and tissues [14–18]. In this thesis we confine our attention to its use in cellular biology.

Certain substances emit radiation when irradiated with a higher intensity light, such as ultraviolet (UV), blue or green, which is of a longer wavelength than that of the exciting light, this is known as *Stokes' Law*. This phenomenon is known as *photoluminescence* [19–22]. There are two types of photoluminescence. If emission persists at an appreciable level after the exciting light is turned off, then we call this *phosphorescence*. If emission persists only so long as the exciting light is on, then we call this *fluorescence* [19, 23].

The first observance and publishing of fluorescence is credited to Sir John Frederick William Herschel around 1852. In 1852, Sir John George Stokes published a 100 page treatise about this luminescent phenomenon and coined the term *fluorescence*, over Herschel's *dispersive reflection*, when he observed that the mineral *fluorite* emitted red light when irradiated by ultraviolet (UV) light [15, 24].

In the remainder of this chapter we present the underlying principles of fluorescence, how specimens are fluorescently marked, the optical principles of microscope design, image acquisition, image processing and common analysis in cellular biology. We only go so far in depth as to present a rudimentary understanding of fluorescence microscopy as is necessary for the comprehension of this thesis.

2.1 Physics of Fluorescence

Why is it necessary to understand the principles of fluorescence? Simple description of the fluorescence process. A more in-depth explanation of the fluorescence process and why stuff goes wrong e.g. phosphorescence, fading, etc. Fluorescence microscopy is a cross-disciplinary field. It is encouraged, if not necessary, to have a photophysical understanding of the principles of fluorescence, for biologists and computer scientists. The knowledge of the "under the hood" mechanics of fluorescence empower computer scientists to make informed and directed research in terms of image processing. The aim here is to present an elementary introduction into the physics of fluorescence.

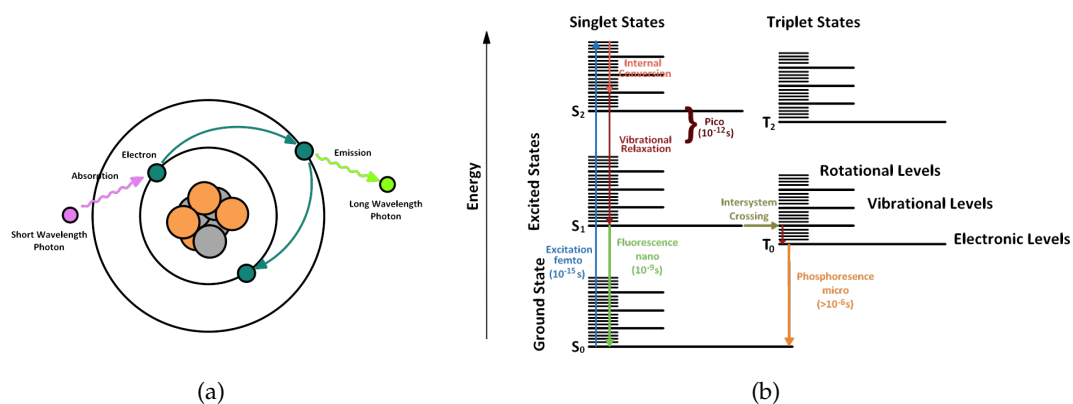


FIGURE 2.1: **(a)** Simplified fluorescence process. **(b)** The Jablonski diagram depicting the electronics states from photon absorption to photo emission.

The overall principle of fluorescence can be explained in three steps [25] as illustrated in 2.1(a): (1) Energy is absorbed by an atom upon collision with a photon. (2) The atom becomes excited and an electron jumps to a higher energy level. (3) Shortly after jumping to the higher energy level the electron returns to the ground state and emits a photon.

Excitation and emission One of the best ways to visualise the fluorescing process is using a Jablonski diagram. A detailed Jablonski diagram is shown in 2.1(b). When a photon collides with an atom, all its energy is transferred to the atom. The energy of the photon is inversely related to its wavelength, $E = h\frac{c}{\lambda}$, where h is Planck's constant and c and λ are the speed and wavelength of light in a vacuum respectively. This increase in energy moves an electron from the ground state S_0 to a higher level energy state. Depending on the energy of the photon and the number of photons absorbed by the photon the electron can move to different energy levels e.g. S_1, S_2 , etc. This process happens almost immediately in the order of femtoseconds (10^{-15} s). Before moving to the lowest next higher energy level (S_1), some energy is lost due to internal conversion and vibrational relaxation. When the electron spontaneously decays from S_1 to S_0 it emits a photon of longer wavelength than the absorption photon. This happens few nanoseconds (10^{-9} s) after excitation.

The difference in wavelength of the emission photon and excitation photon is known as the *Stokes' Shift* or *Stokes' Law*. The larger the Stokes' Shift the easier it is to separate the emission light from the excitation light [14]. The emission curve is often a mirror image and shifted to longer wavelengths than the excitation curve as illustrated in Figure 2.2.

Fluorophores Substances that exhibit the fluorescent property are called fluorophores, also known as *fluorochromes* or *fluorescent dyes*. Early investigations showed that many substance naturally possess fluorescent properties, such as minerals, crystals, resins, crude drugs, butter, chlorophyll, vitamins, etc [14]. This is known as *autofluorescence*. Substances that do not fluoresce must be *stained* such that it can be observed through a fluorescent microscope, more on this later.

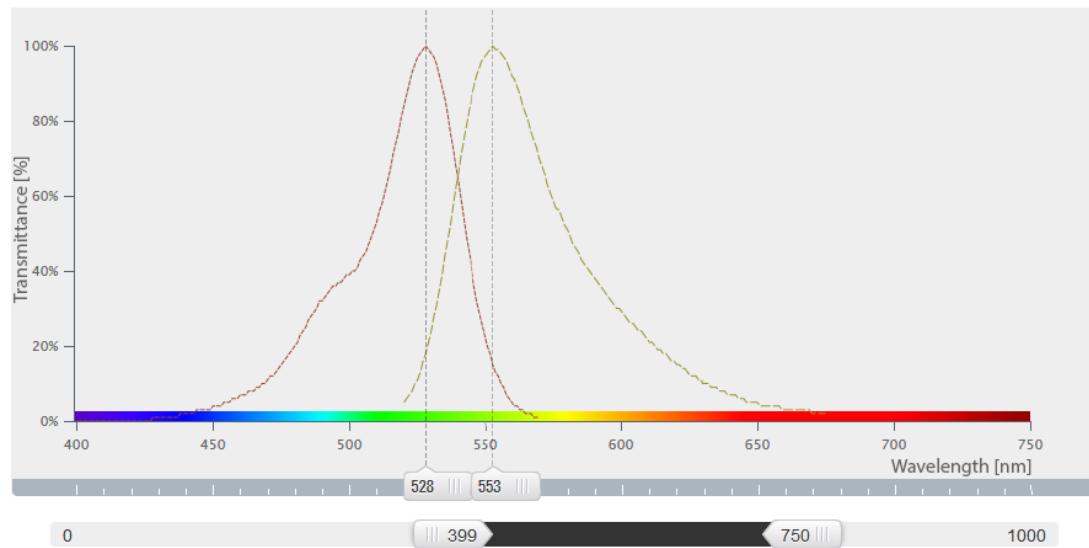


FIGURE 2.2: Normalised Excitation and Emission Spectra of the Alexa Fluor 532 fluorophore. The emission maximum is at 553nm which is a more yellow-green than excitation maximum at 528nm . This image was generated using FluoScout™ web application by Leica Microsystems for determining the optimal fluorescence filter cube set.

Intersystem crossing If the electron spin as the electron transfer between energy states is preserved then the energy states are known as *singlet states*. It is also possible for the electron to reverse its spin. This is very unlikely and is known as *forbidden transition* in Quantum mechanics. When this happens the electron is said to be in a *triplet state*, see 2.1(b). The only way for the electron to reach the ground state is to again undergo another forbidden transition which is unlikely. When this does eventually happen the electron may emit a photon and this phenomenon is known as *phosphorescence*. This process takes much longer, in the order of microseconds (10^{-6}s).

2.2 Specimen Labelling

Why do specimens have to be stained? What is staining? Many of the components of interest, such as cell nuclei, cytoplasm, genes, chromosomes, proteins, do not possess a high degree of, if not any, autofluorescence. In this scenario, these components can be marked with a fluorescent dye [26], also known as a fluorophore or fluorochrome, a substance that can bind to a specific target whose excitation and emission spectra are well known. This is known as staining [15, 16, 24]. Once the specimen is stained it can be indirectly observed using a fluorescence microscope.

What are the most common staining protocols? The most prevalent staining techniques are fluorescence in-situ hybridisation (FISH) and immunostaining [15, 17, 27, 28].

FISH staining **What is FISH? What is the FISH staining techniques used for?** FISH is a molecular cytogenetic technique that uses fluorophores that bind to selected regions in nucleic acids [15, 17]. FISH is the most frequently used staining technique used

primarily for visualisation and localisation of nucleic acid sequences, chromosomes, cytoplasm or organelles which contain those acids [16]. This makes FISH highly attractive for finding specific features in DNA and RNA used in genetic diagnosis and research, medicine and species identification [17, 29]. Figure 2.3(a) is a capture of mouse chromosomes using the FISH staining technique.

Immunostaining What is Immunofluorescence and the two main types, what is the difference between the two, and which is more common? What is the Immunofluorescence staining techniques used for? Immunofluorescence is the detection method where an antibody is used to detect an antigen in a tissue or a cell using fluorescence. Fluorophores are usually conjugated onto antibodies, which are proteins that are designed bind to specific antigens, target proteins, on a cell [30]. The two types of immunofluorescent detection are immunocytofluorescence (ICF) and immunohystofluorescence (IHF). It must not be confused with immunocytochemistry (ICC) and immunohistochemistry (IHC). *Immuno* refers to the immunological technique, i.e. the binding of antibodies to antigens. *Cyto* refers to cells, i.e. cells without the extracellular membrane. *Histo* refers to tissue i.e. cells with the extracellular membrane. *Chemistry* refers to the chemical method of detection, e.g. a change in colour. *Fluorescence* detection by emission of light [31]. Figure 2.3(b) shows the detection of the p53 Binding Protein 1 in perfusion fixed frozen sections of rat kidney.

Live-cell staining FISH and IHC cannot stain live cells. Why? How can we stain live cells? The previously discussed staining techniques are not suitable to observe living cells. The fluorescent dyes used are phototoxic and cause cells to die. The circumvent this problem an elegant solution has been devised. Instead of staining, the cells are modified to produce a fluorescent substance in the target structures. Derivatives of the *green fluorescent protein* (GFP), isolated from the *Aequorea victoria* jellyfish [17, 26, 32], are used as it generates a strong photon emission and is non-toxic to living cells [15, 16, 24].

Important notes about fluorophores and the impact on image quality?

2.3 The Epifluorescence Microscope and Image Acquisition

What is a fluorescent microscope? Schematic layout of a fluorescence microscope? Function and purpose of each component in the fluorescent microscope? A fluorescence microscope is an optical microscope that is designed specifically to exploit the principle of fluorescence to allow for the observation of fluorescently labelled specimens [16, 17, 21, 24, 33]. There are many types of fluorescent microscopes available but the favoured type among many biologists and geneticists is the epifluorescent microscope [22, 34]. The schematic of the epifluorescent microscope is illustrated in Figure 2.4.

Light source What sort of light needs to be generated? What sort of lamps are used? Advantages and disadvantages of certain lamps. The light source is typically a high-luminance light source e.g. Mercury or Xenon arc lamps, LEDs, lasers, etc [15, 16, 34–36]. The primary criterion for choosing a light source is that its characteristic peaks

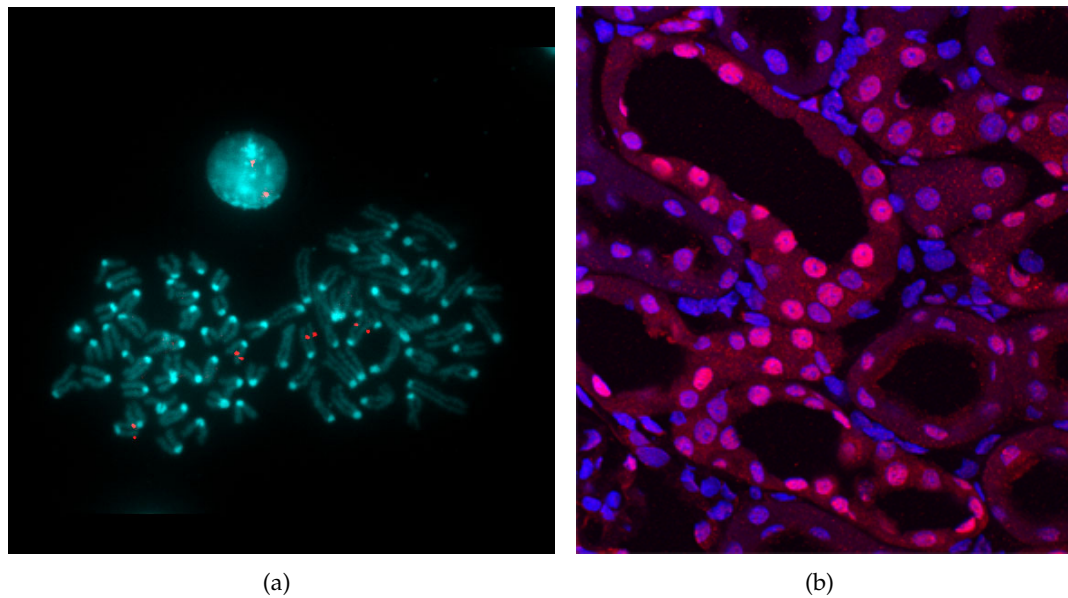


FIGURE 2.3: **(a)** FISH (Fluorescent 'in-situ' Hybridization) in mouse chromosomes using a BAC clone labeled with Spectrum Orange. The picture shows two metaphases and one interphase with two signals in each exemplifying a homozygous mouse for a transgenic clone. Image Source: "All About the Human Genome Project" Genetic and Genomic Image and Illustration Database. **(b)** p53 Binding Protein 1 (53BP1) was detected in perfusion fixed frozen sections of rat kidney using Goat Anti-Human 53BP1 Antigen Affinity-purified Polyclonal Antibody (Catalog # AF1877) at 15 $\mu\text{g}/\text{mL}$ overnight at 4°C. Tissue was stained using the NorthernLightsTM557-conjugated Anti-Goat IgG Secondary Antibody (red; Catalog # NL001) and counterstained with DAPI (blue). Specific staining was localized to nuclei of epithelial cells in convoluted tubules. Image Source: R&D Systems' IHC image database.

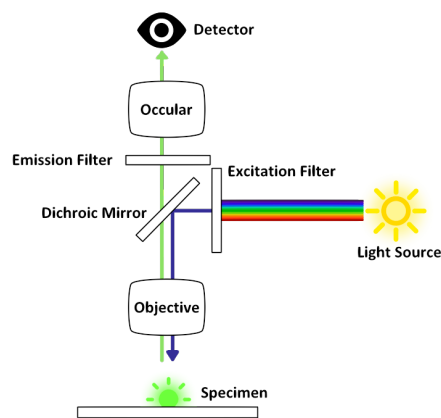


FIGURE 2.4: The schematic of the epifluorescence microscope.

must coincide with the excitation spectrum of the fluorophores being used [14, 17, 32]. Wavelength coverage spans from near infra-red to UV. Mercury and Xenon arc lamps are expensive, an inexpensive and lightweight alternative is bright LEDs [17, 19, 24, 35].

Excitation filter What is an excitation filter? Why is it needed? The incoming light

from the light source is typically multispectral [23]. The excitation filter is a wavelength selection filter which is placed in the path of the incoming light and filters through only those wavelengths in the absorption spectrum of the fluorescent dye [14–17, 24, 30, 32, 36].

Dichroic mirror *What is a dichroic mirror? Why is it needed?* Also known as a *dichroic beam splitter*. This is placed at a 45° angle and reflects the short-wavelength light filtered through the excitation filter at a 90° angle towards the specimen [14–16, 30] and allows the long-wavelength light from the fluorescing specimen to pass through to the detector [19, 32], thus serving as a separation filter between the absorption and emission light [17, 24].

Objective *What is the objective? Why is it needed?* The incoming light reflected of the dichroic mirror passes through the objective lens before reaching the specimen [14–16, 32]. Emission light from the fluorescing specimen is gathered in the objective lens and passed through to the dichroic mirror.

Specimen *Say something about the specimen, for wholeness sake.* The specimen is irradiated by the incoming light from the objective and emits long-wavelength light in all directions. The specimen is stained with a fluorophore whose absorption and emission curves are well known. This is important since the light source and the interference filters are chosen using the peaks of these curves.

Emission filter *What is an emission filter? Why is it needed?* Also known as a *barrier filter* [14, 19, 32]. The light coming from the specimen contains multiple wavelengths and the dichroic mirror is used to filter out the shorter wavelength light. The emission filter is further used to filter out the wavelengths that correspond to the emission wavelengths of the fluorophore [15, 16, 23, 30, 36].

Detector *What is the detector why is it needed?* The detector is used to capture the emission light and can further digital form the image. The detector is usually a photodetector such as a CCD (charge-coupled device) camera or a photomultiplier tube [14–16, 32, 37]. It is vital that an appropriate detector be chosen as this has direct impact on image quality [17].

Other Types of Fluorescence Microscopes: Confocal, TIRF. Acquisition: CCD, Hardware setup effect on image quality, Numerical Aperture, Sub-diffraction The type of fluorescence image data that needs to be captured is application dependant and this impacts the decision on which type of microscope to use. The *widefield*, or conventional, microscope produces 2D image data. 3D image data cannot be captured directly. Instead, a series of 2D images are captured to form a 2D stack. The 3D image is then constructed in software. In this scenario, the most common choice of microscope is the *confocal laser scanning microscope* (CLSM). This microscope system is expensive and acquisition is slower. An economical alternative is the *confocal spinning disk microscope*. To detect single molecules, the favoured technique is *total internal reflection fluorescence* (TIRF) which can be achieved by a modification of the epifluorescence microscope.

2.4 Image Processing

Limitations in Fluorescence Imaging. Preprocessing: Point Spread Function deconvolution, etc. Segmentation

Due to the physical nature of the fluorescence and image acquisition process, there are many factors that can degrade image quality. There are measures that can be taken to largely mitigate some problems but they can never be completely abated. The presence of these factors directly affect segmentation accuracy and subsequently higher level analysis. Therefore, images are processed prior to segmentation to suppress the artefacts and reconstruct the original data [15] or better yet enhance it. Chapter 3 takes a step in this vain. Here we present some of the commonly occurring factors that reduce image quality and the methods used to mitigate them.

Uneven illumination What causes uneven illumination? What techniques are done to reduce it's effect. Vignette effect, etc

Fading What causes quenching and photobleaching? What techniques are done to reduce it's effect. Why is photobleaching worse?

Image distortion What causes image distortion (PSF)? What techniques are done to reduce it's effect. Image distortion occurs primarily due to two factors: The *point spread function* (PSF) and noise [21]. Image formation can be approximated by

$$O = n(s \otimes h), \quad (2.1)$$

where O is the formed image, n is the noise function, s is the exact image and h is the specific PSF of the optical system. We discuss the PSF first. The observed image isn't an exact representation of the real object. Optical systems have an inherent property called the point spread function which is the systems optical response to a point light source [15]. The final image is dependant on the spatial position of a point, numerical aperture (NA) and furthermore differs for various emission wavelengths [16, 38]. The final image is a superposition of all points in the illuminated volume where the contribution of each is described by the PSF.

Theoretically the exact image can be obtained by deconvolution of the observed image with the PSF. However, there are secondary factors that prevent this. Deconvolution seeks to reconstruct the original image given the PSF and certain assumptions about noise [38]. This is an *ill-posed problem* as little is known about the specific PSF or the noise model. Additionally, if the image is corrupted by too much of noise, deconvolution might still produce unsatisfactory results. The PSF is generally experimentally determined and so the true PSF is never attained. Hence, the original image can never be attained by deconvolving with an approximated PSF. The image formation and restoration process is illustrated in Figure 2.5.

Deconvolution has recieved a lot of attention in the passed with very creative approaches [39–43]. Fluorescence microscopy deconvolution is still a very active field of research. Bayesian methods have become popular in fluorescence microscopy image deconvolution, however noise in low SNR imaging conditions still poses a challenge [44]. Recent trends in 3D deconvolution in widefield microscopy use blind depth-invariant of the PSF [45]. Most PSF deconvolution systems naïvely assume depth-invariance however, the PSF changes significantly along the optical axis. There are also

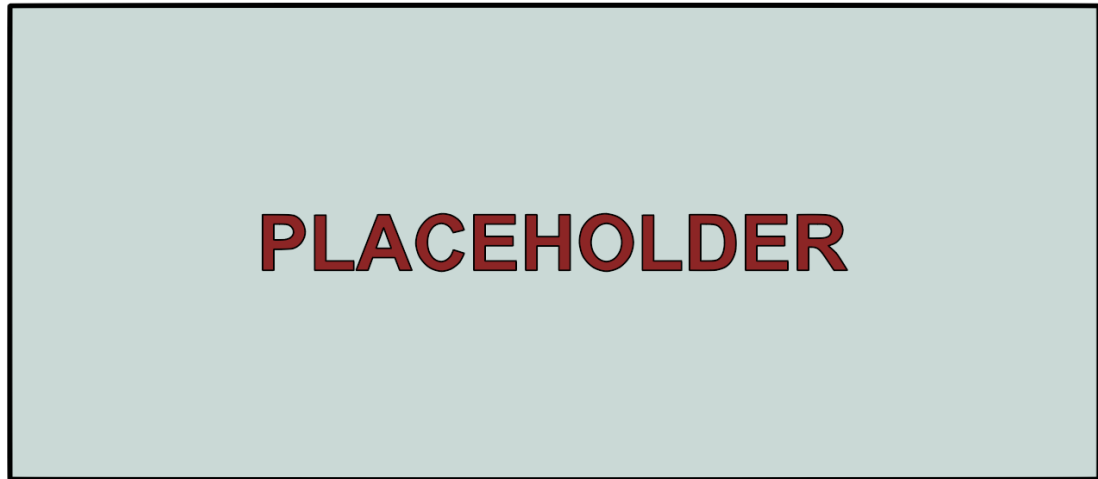


FIGURE 2.5: The image formation process

deconvolution methods that can preserve detail and possibly enhance image quality in diffraction-limited/superresolution imaging modalities [46].

Noise What types of noise are most prevalent? How do they arise? What techniques are done to reduce its effect.

Low signal-to-background contrast What causes the background not to be as black? What techniques are done to reduce its effect.

2.5 Object Measurement and Analysis

What is the purpose of object analysis in FM? What is measured? The aim in fluorescence image analysis is to measure specific properties of interest which enable higher level decision making. Typically, these properties are quantitative measures. In this section we review some of the important quantitative measurements in digital image analysis. It is important to note that for some of the properties of interest, the accuracy of the measurements depend heavily on the accuracy on the segmentation. The properties of interest are application dependent, one might require just the object morphology of structure and hence properties like perimeter, area, shape, intensity, colour, etc are of significance. Alternatively, if one requires the colocalisation of cells, then distance discriminants, such as Euclidean distance, Manhattan distance, Chessboard distance, etc, are of significance [15, 47].

Object measures can be loosely classified into four categories: geometric measures, histogram-based measures, intensity based measures and temporal measures. One can also argue a fifth category statistical classifiers although this is generally used in higher level analysis.

Size measures Perimeter, area and volume are common measures to describe the size of objects. Area and volume are suitable measures to describe the general size of an object. The perimeter of an object is distinctly useful in discriminating its shape complexity. Complex and irregular shapes need a larger perimeter to enclose its area.

Pose measures This measure defines an object's location and orientation. The centroid is used as an object's locale and its orientation is the measure of the angle subtended by its major axis.

Shape measures Shape features are used to distinguish objects from one another. These measures are generally translationally, rotationally and scale invariant and can be used independent of, or in conjunction with, the size measures. Commonly assessed shape parameters are thinness ratio to describe the regularity of an object, rectangularity, circularity, Euler number, moments, central moments, object dispersion, rotationally invariant moments, Zernike moments and elongation.

Shape descriptors Shapes descriptors provide a more wholesome way of describing an object's shape than compared to the single parameter shape measures. Differential chain codes, and its two most common descriptors boundary chain code (BCC) and differential chain code (DCC), are used to represent the distance around an object. Fourier descriptors is another object distance measure that exploits the periodicity of BCC. There are also graph representations of which the two most common are minimum spanning tree (MST) [48, 49] and Delaunay triangulation (DT) [50, 51].

Distance measures There are many ways to compute the separation between objects. The most commonly assessed distance measures are Euclidean distance, Manhattan distance (also known as the City-block distance or absolute value metric), which is a more computationally efficient approximation of Euclidean distance, and the Chess-board distance (also known as the maximum value metric) [52, 53].

Intensity measures Images are segmented generally into region with low intra-region intensity distribution and high inter-region intensity distribution [54, 55]. Common intensity measures are integrated optical density (IOD) [56, 57], is simply the sum of all the gray levels that compose the object, its a reflection of the object's "mass" or "weight", average optical density (AOD), is the IOD divided by the objects area, and contrast.

Histogram measures These measure provide a measure of an object's intensity distribution. Common histogram-based measures are mean, standard deviation, skew, entropy and energy [58, 59].

Texture measures In image analysis texture refers to the spatial arrangement of gray level values [60] and hence a texture feature quantifies some characteristic of the intensity variation within an object. Common texture measures are statistical texture measures, gray-level co-occurrence matrix (GLCM) [61, 62] and power spectrum features [63, 64].

Ratiometric measures Some fluorescent dye respond to the changes in Calcium and Hydrogen ion concentration by changing its spectral properties of the fluorescent emission bands. In this case, a ratio of the intensity can be used to calculate concentration of calcium or pH value [24].

Temporal measures Considering the time domain, many interesting properties can be observed. Commonly computed properties of interest are motility [65–67], like velocity and acceleration, rate of growth, rate of change of colour, etc.

These measures are used in higher decision making processes such as the evaluation of a hypothesis to detect the presence of a certain disease. They are also used to aid in the understanding of biological mechanisms, events and interactions [15].

Chapter 3

Pre-Processing and Post-Processing Scheme for Fluorescence Microscopy Images

3.1 Removal of Artifacts using Connected Components

3.2 Anisotropic Diffusion

3.2.1 Coherence Enhancing Diffusion

3.2.2 Coherence Enhancing Diffusion with Optimised Rotational Invariance

3.3 Poisson Denoising

3.3.1 Total-Variation Denoising

3.4 Contrast Enhancement

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