

Our Waves (Minus Ultrasound)

Type of radiation	Kind of absorption and effect	Interaction
Radio	Penetrates freely & causes almost no biological damage	None
Micro waves	Large frequency, cause heating	None
Infrared	Penetrates a bit & causes general heating, causes tanning	None
Visible	Penetrates several cm into skin, causes tanning	None
Ultraviolet	Very low penetration of skin, causes sunburn & skin cancer	Strong absorption, causes photochemical reactions, causes mutations, causes cancer
X-rays and Gamma rays	Penetrates completely, causes ionization	Strong absorption, causes ionization, causes mutations, causes cancer

Spatial Resolution – Image of a Point is Distorted

Fluorescence Microscopy – Contrast from Color

Example of Class Approach – Understanding Essence of Confocal Microscopy

Medical Imaging

- Ultrasound
- [2D-B-mode](#)
- Projection Radiography/CT
- [Radiography: Essence](#)

Medical Imaging

- Nuclear Imaging
- [Nuclear: Essence](#)
- MRI

Course Approach – Principles & Practice of Imaging

- Radiography (Projection & Tomographic)
- [AT1.1 Transmission Imaging \(Projection and Tomographic\)](#)
- MRI
- [AT3.3 Diagnostic MRI](#)

Review of Waves Basics

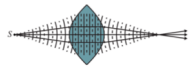
- Spherical waves are produced by, or collapse into, point sources
- Spherical become planar with distance
- Wave fronts where the wave phase (commonly magnitude) is constant often are depicted
- Lines (rays) along the direction of energy propagation and perpendicular to wave fronts also often are depicted

Lenses and Imaging

- Object is a collection of sites that scatter/emit electromagnetic waves
- Lens is a refracting device that reconfigures energy to produce "image" of object
- [Circular Waves](#)
- [Demonstration](#)

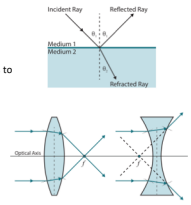
Focusing a Diverging Wave – Part 1

- Wave propagation slows in a substance like glass, i.e., $v = c/n$, where c is speed in vacuum and n is refractive index
- Bulging/convex lens intercepts and slows center before periphery reducing divergence
- Lens release edges before center converting plane into converging



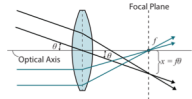
Focusing a Diverging Wave – Part 2

- Rays bend at interfaces with different n 's
- Bulging lens refracts upper rays down at both interfaces and lower rays up at both interfaces to focus



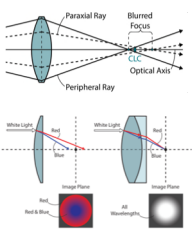
Attributes of Focusing

- Lens brings parallel rays to focus at a "point" in focal "plane" at a distance f from center of simple lens
- If θ is a small nonzero angle the focus is off axis at distance $s = f\theta$
- Focal point determined by angle of rays with respect to optical axis → lens maps angle into position



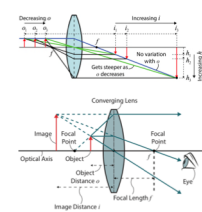
Aberrations

- Aspheric (e.g., hyperbolic) lenses focus all light to a point
- Hard to fabricate → use spherical lenses
- Light waves passing through the periphery and the center do not focus together (spherical aberration)
- Different wavelengths do not focus together (n varies with wavelength – chromatic aberration)
- Objective (main imaging component in microscope) contains 15-20 lenses to correct aberrations



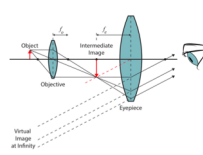
Attributes of a Converging Lens

- Real images produced as long as object positioned beyond focal length
- Magnified or minified based on object position
- Magnified virtual image produced if object within focal length – mechanism underlying simple magnifier



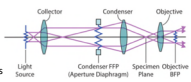
Simple Compound Microscope

- Object just outside focal point of high power objective lens
- Real magnified (4 - 100x) intermediate image produced just inside/at focal point of eyepiece lens
- Intermediate image is object for eyepiece
- Magnified virtual image produced at ∞ and visualized by eye



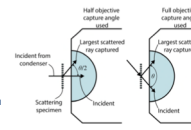
Transmitted Light (Kohler) Illumination

- Illuminator usually an inhomogeneous tungsten/halogen filament
- Want uniform illumination of object
- Collector lens focuses illumination in front focal plane (FFP) of condenser lens
- Parallel (uniform) illumination exits condenser and hits object
- Illumination collected by objective and focused in its back focal plane (BFP)



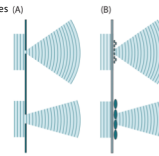
Effects of Condenser on Resolution

- Condenser creates uniform illumination at specimen
- Condenser also increases ray capture/resolution
- Without oblique illumination, only half the objective capture angle is used
- With oblique illumination, can use full capture angle of objective



Wave Optics - Diffraction

- Diffraction occurs when light encounters obstacles (A)
- Objective aperture and λ -sized objects in specimen are two key sites of diffraction



Effects of Diffraction – Resolution

- Aberrations small → resolution is diffraction-limited
- Key effects of diffraction:
 - Image of self-luminous point is diffraction (Airy) pattern created by circular aperture
 - Small objects all have same apparent size
 - $\sigma = \lambda$
 - Close objects blur together
- Minimize diffraction with high NA oil-immersion objective
 - Want big objective aperture and oil to bend light toward aperture → big NA = $n \sin \theta$
 - Good objective NA = 1.4 with $n_{oil} = 1.5$ and collection angle $\sim 70^\circ$
 - $r_{max} = 0.2 \mu m$

Image Formation Based on Diffraction

- Diffraction occurs when illumination interacts with small particles or features in specimen
- Diffraction and absorption make specimen visible
- Image formation depends on diffraction of light into divergent waves and subsequent recombination of light into magnified image through interference

Abbe Theory of Image Formation

- Abbe analyzed image formation using a grating as specimen
- Abbe demonstrated that a series of orderly diffraction spectra appear in BFP of objective
- Specimen (grating) spacing and diffraction spectra spacing inversely related
- Diffacted waves propagate beyond BFP and interfere with undiffracted to generate inverted image in image plane
- Unresolved image if only $m = 0$ (undeviated light) captured → diffraction essential

Diffraction Patterns in BFP of a Simple Lens

- Illuminate grating with laser light
- Insert lens f away from grating
 - Diffraction pattern visible in BFP
 - Image of grating in image plane at $2f$
- Equivalent view
 - Lens performs a Fourier Transform on object – produces transform in BFP
 - Light in BFP propagates onward to produce image in image plane

Abbe Theory – Implications for Resolution

- Must capture at least two orders in diffraction pattern (e.g., $m = 0, 1$) to generate an image
- Capturing more orders improves resolution
- For grating under planar illumination diffraction orders satisfy
 - $m\lambda = d \sin \theta$ (d = grating spacing)
 - To resolve a spacing d in specimen using an immersion medium of refractive index n requires $\lambda/n = d \sin \theta$ (setting $m = 1$)
 - $d = \lambda/n \sin \theta = \lambda/NA$ is smallest resolvable spacing.
 - If illumination oblique $d = \lambda/2NA = \lambda/2$

Manipulating an Image by Spatial Filtering in the BFP

- Image contrast and quality can be altered by manipulation of undiffracted and diffracted beams in BFP
- Known as spatial filtering
- To implement spatial filtering need to understand relationship between diffraction pattern and image/object attributes
 - Central spot in pattern (undeviated light) produces homogenous background in image plane
 - More central spots in pattern encode coarse details in object
 - Peripheral spots in pattern encode fine details and sharp edges in object
- Example - filtering out spots at large distance from center results in a loss of image detail

Interactive Tutorial - Predicting Effects of Spatial Filtering

- With aperture closed, diffraction from a line grating produces orthogonal line of aperture replicas in BFP of objective
- Filter out $m = 1$ from BFP
- Image = line grating with $1/2$ the spacing
- This was one of Abbe's most surprising observations

An Aside on Coherence

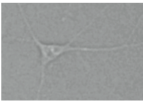
- Much of past discussion implicitly assumed illumination has coherence
- Diffraction pattern visible in BFP if illumination coherent
- In a microscope, filament illumination is partially coherent and thus diffraction pattern visible in BFP of objective

The Importance of Contrast

- Capturing rays is not enough – need contrast
- Specimens that induce changes in amplitude or λ of a wave have good contrast because eye sensitive to intensity and color
- Staining cells with absorbing or fluorescing dyes therefore generates good contrast
- Staining important in medical diagnostics
 - Identification of cancerous cells
 - Identification of bacterial type

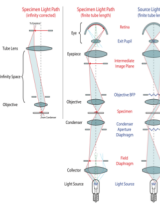
Generating Contrast from Transparent Specimens

- Transparent specimens induce changes in phase of wave and have poor contrast because eye insensitive to phase
- Image contrast and quality can be enhanced by manipulation/spatial filtering of undiffracted and diffracted beams
- Manipulation implemented in BFP of objective and FFP of condenser
 - Understanding Kohler illumination is key



Kohler Illumination and Contrast Enhancement – Part I

- Basic features of Kohler most relevant to contrast enhancement are:
 - Kohler creates two sets of "conjugate planes"
 - Conjugate planes are simultaneously in focus and can be viewed superimposed
 - Sets of conjugate planes are reciprocally related (light in focus in one set of conjugate planes is defocused/parallel in the other set of conjugate planes and vice versa)
 - One set of conjugate planes lies along the imaging path and consists of the field diaphragm, next the specimen, then the intermediate image, and finally the retina
 - Other set lies along the illumination path and consists of the light source, next the condenser diaphragm located in FFP of the condenser, then the BFP of the objective, and finally the exit pupil of the eyepiece

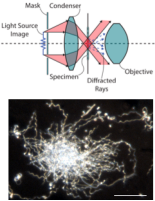


Kohler Illumination and Contrast Enhancement – Part II

- More subtle features of Kohler relevant to contrast enhancement are:
 - The diffraction pattern/frequency transform of the specimen appears in the BFP of the objective (because the specimen and the objective BFP lie in the two distinct sets of reciprocally related conjugate planes)
 - To enhance contrast, the illumination profile and the diffraction pattern of the specimen can be altered at the FFP of the condenser and the BFP of the objective, respectively (reasoning here is similar)
- These attributes figure prominently in the foundations of darkfield and phase-contrast imaging

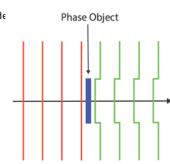
Darkfield – A Simple Method of Visualizing Transparent Specimens

- Analogy for darkfield
 - Stars visible at night (dark background) but invisible during day (bright background)
- Two ways to create dark background
 - Filter out undiffracted light in BFP of objective
 - Place mask in FFP of condenser to create hollow cone of illumination ($NA_{condenser} > NA_{objective}$)
- Mask approach
 - Illumination misses objective if no specimen
 - Specimen diffracts some light into objective → specimen image imposed on dark background
 - <http://www.nyu.edu/classes/phys321/lectures/phys321lec17.html>
- Negatives – bad for thick specimens, sensitive to debris, requires bright illumination
- Positives – good if specimen thin
 - <http://www.oxford-journals.org/doi/abs/10.1093/aob/131.3.401>



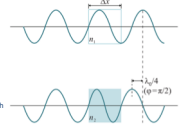
Effect of a Phase Specimen on Wavefront

- Effect of a phase specimen on an incident planar wavefront
 - Plane wave passing through transparent sample typically is retarded in phase
 - Emerging wave has indentation – wave is phase modulated



Important Example – Phase Shifts Induced by Refractive Index Differences

- Speed and wavelength change in a medium with refractive index n
- $\lambda = \lambda_0/n$
- Wavelength/speed differences in media of different n cause waves to get out of phase
- Example at right
 - Waves out of phase by $\lambda/4$ due to wavelength difference
 - Phase shift is $\pi/2$
- In general, $\varphi = \frac{2\pi}{\lambda_0} (n_2 - n_1) \Delta x$

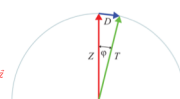


Analyzing Phase Contrast Using Vector Diagrams

- The emerging total (T) wave produced by a thin, transparent cell is the sum of a weak, diffracted (D) wave and a zeroth-order/undiffracted (Z) wave $T = Z + D$
- Diffracted wave is what is observed in Darkfield when Z is blocked
- Represent each wave using a vector with length proportional to wave amplitude and orientation reflecting wave's phase shift relative to Z

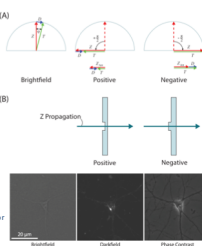
Brightfield Diagram for a Thin Phase Specimen

- Phase Specimens
 - No absorption so Z and T have same length
 - D related relative to Z due to specimen-induced phase shift
 - D is small and 90 degrees out of phase with Z
 - Specimen is invisible because $|T| = |Z|$ (no amplitude modulation)



Phase Contrast – A Nobel Prize-Winning Method of Visualizing Transparent Specimens

- Zernike made phase effects visible by spatial filtering in BFP to alter Z
- Positive Phase Contrast
 - Z wave advanced by 90 degrees → Z and D antiparallel (interfere destructively)
 - Specimen dark against bright background
- Negative Phase Contrast
 - Z retarded by 90 degrees → constructive interference
 - Specimen bright against dark background
- Implementation
 - Insert spatial filter in BFP of objective
 - Filter could be small etch or bump in glass
 - Ideally only Z passes through filter
 - Width, refractive index, and absorbance of filter adjusted so Z attenuated and retarded or advanced by 90 degrees
 - Amplitudes of Z and T now differ



Phase Contrast with Annular Illumination

- Brighter image obtained with broad annular illumination (A)
- Annulus/ring placed in FFP of condenser
- Matching phase plate in BFP of objective
- Z shifted and attenuated by annular phase plate
- Interactive tutorials
 - www.chemeddl.org/chemeddl/viewContent.do?cid=123456789
 - www.scribd.com/document/123456789/Phase-Contrast-Microscopy
- Advantages of Phase Contrast
 - Visualize living and transparent specimens without staining
- Disadvantages
 - Specimen should be thin
 - Image artifacts (e.g., halos)

Using Polarized Light to Generate Contrast

- What is polarization of light?
 - Light is composed of electric and magnetic fields
 - Electric field vector defines the direction of polarization
- Types of polarization
 - Natural light randomly polarized
 - Plane wave linearly polarized (fixed direction of electric field)
 - Two phase-shifted, perpendicular plane waves can combine to produce elliptically polarized light
 - Circularly polarized light: the direction of the electric field rotates

Generating and Manipulating Polarized Light

- Linear polarizers used to generate polarized light
 - Output is polarized along transmission axis
 - Output intensity depends on alignment between input polarization and transmission axis
- Randomly polarized light incident on crossed polarizers ⇒ no output
 - Crossed polarizers used to generate dark background in polarization microscopy
 - Intervening, third polarizer can rotate the polarization and create a signal against a dark background

Polarized Light Microscopy

- Used to create images of anisotropic (birefringent) specimens
 - Crystals, oriented biological specimens are birefringent
 - Light polarized along one direction preferentially slowed (encounters a higher refractive index)
 - Different refractive indices can lead to "double refraction"
- Mechanism of contrast generation
 - Crossed polarizers in microscope generate dark background
 - Specimen creates perpendicular \parallel and \perp waves that get out of phase
 - \parallel and \perp generally combine to produce elliptical light that partially passes second polarizer to create signal against dark background

Nomarski/DIC

- Phase contrast probes magnitude of phase/optical path length (OPL)
 - OPL incorporates the effect of path length and refractive index on phase shift
- Nomarski probes gradients in optical path length
- Essence of Nomarski
 - Generate two slightly displaced (laterally sheared) independent beams that create a "double image"
 - Each image is an imprint of the specimen-induced phase lag
 - Beams ultimately reverse laterally shifted (unsheared) and two images superimpose out-of-register
 - Unshearing generates phase differences in object regions containing OPL gradients, such as edges
 - These regions generate elliptical light that can pass an analyzer, similar to polarization microscopy

Phase Contrast Versus Nomarski

Characteristics of Phase Contrast	Phase Contrast	DIC	Halos	Axial Resolution	Phase
Image Brightness (Significant at 10x Percent)	1.3 Percent	0.36 - 2.3 Percent			
Latent Resolution	Condenser Annulus Restricted	Superior			
Axial Resolution (Depth Discrimination)	Poor	Superior			
Illuminating Aperture	10 Percent of Objective NA	Variable			
Phase Shift Detector Link	< 1/100	< 1/100			
Halos and Shade-Off	Yes	No			
Stained Specimens	Not Useful	Useful			
Cost	Moderate	High			

Basics of Fluorescence

- Fluorescence = spontaneous emission of light by molecules in an excited state
- Origins of fluorescence
 - Molecules with alternating single/double bonds have mobile electrons
 - Transitions of electrons between energy levels ⇒ absorption or emission
- Insights from Quantum Mechanics
 - Spacing between quantum energy levels determines absorption/emission spectra
 - $|E_1 - E_2| = h\nu = hc/\lambda$
 - Energy spacing related to length of conjugated chain
 - Small molecules absorb/emt at shorter λ

Key Attributes of Fluorescence

- Stokes shift
 - Absorption λ_{abs} > emission λ_{em}
- Jablonski diagram – origin of shift
 - Molecule in a high energy state loses some energy non-radiatively before fluorescing and returning to a low energy state
 - Non-radiative loss ⇒ emission is a lower energy transition (longer λ , than absorption)
 - Tutorial: www.chemeddl.org/chemeddl/viewContent.do?cid=123456789
- Photobleaching
 - After $10^4 - 10^7$ cycles of excitation molecule undergoes a chemical change ⇒ non-fluorescent

Key Attributes of Fluorescence Microscopy

- Sensitive form of microscopy
 - Stoke's Shift facilitates generation of an image superimposed on a dark background
- Images can reveal several specific, distinct components within a specimen
 - Possible by tagging distinct components of a specimen with different, "spectrally compatible" fluorophores
- Living specimens can be studied
 - Tagging often is not toxic
- ~80% of all microscopy investigations in biology are based on fluorescence

The Fluorescence Microscope

Epi (reflected light) fluorescence microscopes most popular

Advantages of epifluorescence

- Objective + condenser so always aligned
- Most excitation travels away from objective
- Can alternate between reflected/fluorescence and transmitted/phase observation

Light Path

- Arc provides broad band illumination
- Excitation filter passes range of λ 's that effectively excite fluorophore
- Dichromatic mirror reflects excitation
- Longer λ emission emitted by specimen and partially collected by objective
- Dichromatic transmits emission
- Emission filter cleans up signal
- Eye or camera detects

Understanding the Dichromatic Mirror

- Dichromatic mirrors also called interference filters
- Based on thin film interference
 - Colors in soap bubbles/oil slicks
- Excitation reflected \rightarrow matches low dichromatic transmission
- Emission transmitted \rightarrow matches high transmission

DAPI-FITC-Texas Red (Triple Band Excitation)

Transmission (Percentage) vs Wavelength (Nanometers)

Imaging Modes – Thin Samples

(A) Single color
(B) Multi-color
(C) Time-lapse

The Blur Problem for Thick Samples – Part I

Image of a point source = point spread function (PSF)

- PSF extends over many microns in the image plane
- Out-of-focus intensity extensively "contaminates" intensity from the focal plane
- Images of thick samples plagued by blur

The Blur Problem for Thick Samples – Part II

- Objective captures out-of-focus fluorescence
- Images of thick specimens degraded by blur
- Two well-established methods of removing blur
 - Confocal microscopy – spatial filtering in the intermediate image plane
 - Image deconvolution – computational deblurring

The Confocal Approach

- Light from a laser focused to a small spot on sample – laser scanning confocal microscopy (LSCM)
- Pinhole in intermediate image plane preferentially rejects fluorescence from out-of-focus planes
- Image of extended specimen reconstructed point by point by scanning x/y plane \rightarrow never visible in eyepiece

LSCM Implementation

Instrumentation

- Laser reflected by dichromatic onto scanning mirrors
- $\sim 0.5 \mu\text{m}$ excitation spot scans over sample
- Objective directs light onto sample and collects emission
- Emission follows same path as excitation then travels to **pinhole** point in space
- Pinhole positioned at this point excludes out-of-focus fluorescence

Three-dimensional Imaging with LSCM

- Collect optical sections to generate a 3D image

Advantages and Limitations of LSCM

- Advantages**
 - Produces optical sections without blur
 - Highly automated
- Limitations**
 - Slow acquisition (~ 1 sec/image)
 - Laser/excitation lines limited
 - Expensive to purchase and operate
 - High intensity exposures cause bleaching
 - Not good for dm^3 samples
- Spinning disk confocal microscopy circumvents some of these problems

Spinning Disk Confocal (SDCM)

- Method of choice for faster subcellular processes in living specimens
- Nipkow disk achieves multi-beam scanning
- Disk in plane conjugate to specimen
 - Contains tens of thousands of pinholes
 - ~1000 illuminated at a time to give multi-beam illumination
 - Fluorescence excited by a pinhole passes back through same pinhole to achieve deblurring
 - Specimen scanned in < 1 ms during partial disk rotation

State-of-the-Art SDCM System

- Yokogawa system**
 - In principle, can capture 2000 frames/second
 - Two spinning disks
 - Upper disk focuses light onto lower pinholes
 - Improves transmission ~10-fold → vastly improved brightness
- Additional strengths**
 - Reduced photobleaching
 - Use of novel ultrasensitive detectors
 - Use of arc lamps
- Weaknesses**
 - Reduced axial resolution (pinhole cross-talk)

The Blurring Function of an Optical System

- Imaging Theory I**
 - Disparity between image and object described by point spread function (PSF/response function)
 - PSF = image of a point object and has significant spatial extent
 - Arbitrary object is a sum of point objects → image is a sum of PSFs
- For diffraction-limited systems, the PSF is a 3D diffraction pattern
- The PSF also can be measured experimentally to determine how microscope distorts object
 - Collect 3D images of sub-resolution fluorescent beads

Removing Blur by Deconvolution

- Imaging Theory II**
 - Image = Object + PSF
- The major source of blur is capture of fluorescence from out-of-focus fluorophores
- David Agard
 - Deconvolution gets the photons back where they belong

Super-Resolution Imaging

Confocal and deconvolution reduce blur but do not significantly enhance resolution beyond the diffraction limit

Two (multi) photon microscopy another option well suited for deep imaging

Super resolution imaging techniques can achieve markedly enhanced resolution

- Total internal reflection fluorescence (TIRFM)
- Structured illumination (SIM)
- Photoactivation localization (PALM)
- Stimulated emission depletion (STED)

Two- (Multi-) Photon Microscopy – A Method of Deep Imaging

- Fluorophores do not see two or more photons at the same time unless excitation very intense
- Very bright excitation light can excite a fluorophore using two or three low energy (infrared - IR) photons
 - Two 800 nm photons can induce same transition as one 400 nm
 - Three 1020 nm photons can induce same transition as one 340 nm
- Low energy photon puts molecule in a virtual state (violates energy conservation but exists transiently via Heisenberg Uncertainty)

Multi-Photon – Implementation and Outcome

- Absorption ∝ (Intensity)² → need a focused, pulsed laser
- Excitation and emission confined to a very tight region near laser focus → no out-of-focus fluorescence generated
- Advantages
 - No out-of-focus fluorescence → no pinhole
 - Excitation confined to focal plane → little photobleaching
 - Reduced scattering problems and good IR photon penetration → clear images of deep structures
 - Few endogenous molecules absorb IR → low toxicity
- Disadvantages
 - Need bright IR sources
 - H₂O absorbs IR → heating
 - Need to scan
 - Resolution slightly worse than confocal

Total Internal Reflection Fluorescence – A Method of Surface Imaging

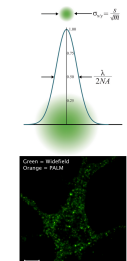
- Geometrical optics**
 - Law of reflection → light incident on medium of lower refractive index bends away from normal
 - At critical incidence angle, light bends to 90 degrees → totally reflected
- Wave optics**
 - Evanescent electric field penetrates ~100 nm into lower index medium (e.g., cell)
- Axial super-resolution in TIRFM**
 - Fluorophores > 100 nm from interface are not excited
 - Markedly reduced out-of-focus contamination
 - Imaging restricted to regions near interface

TIRFM – Implementation and Outcome

- Through-objective TIRFM**
 - Laser-based excitation directed at a peripheral position in the BPF of a very high NA objective
 - Peripheral position maps into a high incidence angle, ensuring that the condition for TIR is met
- TIRFM powerful method of probing biological processes near the cell membrane
 - Endocytosis
 - Transport along cell surface
 - Background reduction in super-resolution single-molecule imaging


PALM – Implementation and Outcome

- Illuminate with an intensity that activates ~1% of molecules – separated by $> \lambda/2NA$ (Abbe limit)
 - ◊ Calculate coordinates of all "on" fluorophores in parallel
 - ◊ Center coordinates generated by fitting diffraction patterns to Gaussians
 - ◊ Image until bleaching switches fluorophores "off" and then activate a new subset
 - ◊ Uncertainty in center coordinates is $\lambda/2M$, where $m = \#$ of photons detected
 - ◊ If $m = 100$, resolution enhanced by 10x
 - ◊ Image assembled in a manner similar to painting via pointillum



PALM – Resolution Subtleties

- Image is reconstructed from samples that are spaced d apart
 - ◊ $d =$ observed inter-fluorophore spacing
- Nyquist-Shannon sampling theorem
 - ◊ Reconstructing a certain level of detail (achieving a resolution, R) requires sampling at $d < R/2$
- Implications
 - ◊ Precise localization is not enough
 - ◊ Sufficient sampling is essential
- Sampling theorem very important in imaging
 - ◊ Insufficient sampling leads to reconstruction of incorrect signal frequency
 - ◊ Aliasing = appearance of "false" low frequency in signal due to insufficient sampling
 - ◊ Aliasing = wrong direction of blood flow in Doppler ultrasound

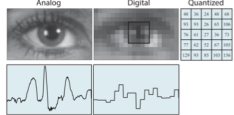


Comparison of Stochastic and Targeted Switching

Stochastic	Targeted
Set up relatively simple	Set up requires expertise
Fluorophore concentration must be high enough for reconstruction	Fluorophore concentration is not a big concern
Illumination must produce on fluorophores separated by $> \lambda/2NA$ Low background is essential	Illumination used for switching often intense and might damage live samples
$m > M$ (threshold) photons yield coordinates of just one fluorophore	All photons from fluorophores in d contribute, so fast scanning can discern small features
Read only where fluorophores present	Read a lot of empty space
Fluorophores switched just once	Fluorophores switched multiple times (fluorophore fatigue)

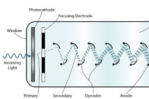
Digital Image Processing and Analysis

- What is a digital image?
 - ◊ Useful to compare with analog image/signal
 - ◊ Analog signals vary smoothly, like a traditional film generated photograph or voltage
 - ◊ Digital signals vary discretely
- Converting an analog image into a digital format
 - ◊ Converts image into 2D array of integers
 - ◊ Involves sampling (taking periodic snapshots)
 - ◊ Quantization of samples with integers (e.g., 0 to 255)
 - ◊ Larger numbers correspond to brighter samples



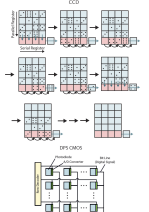
Detectors for Digital Imaging – Part I Point Detectors

- Photomultiplier tube (PMT) – light into current
 - ◊ Very high gain up to 10^8
 - ◊ High signal-to-noise because dark current is very low
 - ◊ Temporal resolution a few hundred picoseconds
 - ◊ Low "quantum efficiency" and spectral response is nonuniform
- Photodiode – light into current
 - ◊ Small-point detector
 - ◊ Can be assembled into an array that is capable of spatial discrimination
 - ◊ Good quantum efficiency
 - ◊ Lacks gain



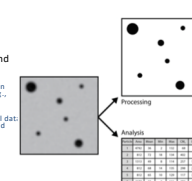
Detectors for Digital Imaging – Part II Spatially Sensitive Detectors

- Cameras – 2D array of detectors (pixels)
 - ◊ Light from object focused onto the camera
 - ◊ Photon-induced charge (proportional to the incident intensity) generated in each pixel
 - ◊ Charge distribution in detector array generates image of object
- Charge-coupled device (CCD) – the standard for decades
 - ◊ Charge is transferred and then converted to a voltage and digitized serially
- Scientific-grade complementary metal-oxide semiconductor (CMOS) device – the new standard
 - ◊ Key features – charge to voltage conversion and, in some cases, digitization occur at each pixel
 - ◊ Individual pixels or a subset of pixels can be read out
 - ◊ Much faster than CCDs



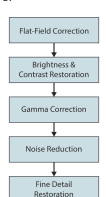
Introduction to Image Processing and Analysis

- Digital images can be visualized, processed, and analyzed on a computer
- Difference between processing and analysis
 - ◊ Processing involves modification of an image to produce another image (e.g., grayscale into black & white)
 - ◊ Analysis involves obtaining numerical data from images, which may be converted into a visual format
- Possible steps in processing and analysis
 - ◊ Image restoration
 - ◊ Image enhancement
 - ◊ Binary conversion/segmentation
 - ◊ Measurement



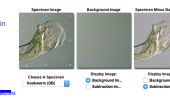
What is Restoration?

- Correcting defects that arise during acquisition from imperfections in the optics, illumination, detector, etc.
- Restoration Strategy



Flat-field Correction & Background Subtraction

- Flat-field correction
 - ◊ Removes degradation from sources like scratches in the optics, detector imperfections, and uneven illumination
 - ◊ Important when image is to be quantified
- Background subtraction
 - ◊ Visually similar results



Brightness & Contrast Restoration

- Brightness measures image lightness
- Contrast measures grayscale or color variation
- Two tools
 - Histogram = graph of intensities in image on x and number of pixels with intensity on y
 - Input-output lookup table (LUT) = function/rule that transforms an input pixel value into a new output
- Histogram stretching
 - Using histograms and LUTs to enhance contrast
 - Map image minimum into zero and image maximum into 255 (for 8-bit) and ramp linearly in between

Gamma Adjustment

- Non-linear (exponential) adjustment of histogram to facilitate simultaneous visualization of both bright and dim features
 - For 8-bit image $I_{out} = 255 * (I_{in} / 255)^\gamma$
 - $\gamma < 1$ enhances differences among dimmer pixels
 - $\gamma > 1$ enhances differences among brighter pixels

Noise Reduction – Part I

- Noise is a random signal superimposed on the true signal
- Reduction achieved using filters
- Spatial convolution filters
 - Multi-pixel operation → output contains contributions from several neighbors
 - Example – smoothing with mean box filter
 - Overlay kernel containing "ones" on center pixel, multiply, add, and replace center by result
 - Mean reduces high-frequency noise but also blurs image
 - Gaussian gives less weight to distant neighbors – reduces noise but with less blur

Noise Reduction – Part II

- Median filter
 - Box around pixel, sort, and replace pixel by median
 - Deals effectively with dramatic noise and better at preserving fine detail than convolutions
 - Computationally intensive

Noise and Resolution

- Diffraction and sampling can affect resolution
- Noise also can affect resolution
 - Noise often causes resolution to be much lower than the diffraction limit
 - Image noise quantified using the signal-to-noise ratio

Fine Detail Restoration

- Enhance detail
 - Example = edges
- Unsharp filter
 - Generate an "unsharp" (smoothed) version of image (e.g., using mean filter)
 - Subtract smoothed from image to remove larger-scale detail and produce an image retaining finer detail
 - Add a portion of "edge" image to original to produce sharpened, edge-enhanced image

Image Enhancement

- A more subjective process directed at altering an image to influence impact on the observer
- One example – local contrast enhancement
 - One popular method, used by Fiji, is contrast limited adaptive histogram equalization (CLAHE)
 - Effect shown at right

Color Processing and Color Coordinates

- Color processing
 - Smoothing, sharpening, etc.
- Hue, saturation, intensity (HSI) better than RGB for color processing
 - Hue = dominant λ
 - Saturation = color purity
 - Intensity = brightness
 - HSI decouples brightness from color
 - Convert to HSI and process just intensity channel to avoid introducing color shifts

Binary Conversion & Image Segmentation

- Binary Conversion
 - Converting grayscale or color image to a 1-bit (black and white) image
 - Used to segment an image into objects of interest and uninteresting "background"
 - Segmentation widely used
- Histogram-based thresholding
 - Simple case – histogram has two peaks for objects and background
 - Set threshold in valley so objects above are white and objects below are black

Image Analysis (Measurement)
– One Important Example –

- **Single particle tracking (SPT)**
 - ◊ Track individual vesicles manually using Fiji
 - ◊ Determine x,y coordinates as a function of time
 - ◊ Determine trajectories, speeds of motor-directed transport, and diffusion coefficients

