Chapter 4 – Microscopy

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4.1 Resolution of Optical Microscopes

- The Microscope can be approximated by 2 lenses $F[F[U[x, y]]]$

- "-" sign means inverted image

- The objective is the most important part of the microscope

- Usually a third lens (ocular) images $F_2'$ at $\infty$, such that we can visualize it with the relaxed eye.
4.1 Resolution of Optical Microscopes

- The objective lens dictates the resolution or size of the smallest object that the microscope can resolve.
- Contrast is generated by absorption, scattering, etc.
- Microscopes can be categorized by the methods that they use to produce contrast.
- Let’s consider an infinitely small object (point):

![Diagram](image)

How small can we see?
4.1 Resolution of Optical Microscopes

- Fourier properties of the lens; the reconstructed field is:

\[
U[x, y] = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} U[\xi, \eta] e^{2i\pi (x_1\xi + y_1\eta)} d\xi d\eta
\]  

(4.1)

- We know that \( \xi < \infty \) and \( \eta < \infty \) because \( \xi^M < \frac{x_M}{\lambda f} \) and \( \eta^M < \frac{y_M}{\lambda f} \).

- We can access only a finite frequency range and therefore we can only achieve finite resolution.

- We would need an infinite spectrum to reconstruct a \( \delta \)-function (in this case a point)
4.1 Resolution of Optical Microscopes

- Given the finite frequency support we can write:
  \[ U[\xi', \eta'] = U[\xi, \eta] \cdot H[\xi, \eta] \]  \hspace{1cm} (4.2)

- Where
  \[ H = \begin{cases} 
  1 & \text{if } \xi < \xi^M, \eta < \eta^M \\
  0 & \text{otherwise}
  \end{cases} \]

- So, eq. 4.1 becomes
  \[ \bar{U}[x, y] = F[U[\xi, \eta] \cdot H[\xi, \eta]] \]  \hspace{1cm} (4.3)
4.1 Resolution of Optical Microscopes

- Use the Convolution Theorem once more (which states that convolution in one domain is multiplication in another) to get:

\[
\overline{U}[x, y] = U[x, y] \otimes h[x, y]
\] (4.4)

- Where \( \overline{U}[x, y] \) is the microscope image, \( U[x, y] \) is the ideal image, \( h[x, y] \) is the impulse response.

\[
h[x, y] = F[H[\xi, \eta]]
\] (4.5)

- \( H[f_x, f_y] = \begin{cases} 1 & \text{if } f_x^2 + f_y^2 \leq w^2 \\ 0 & \text{otherwise} \end{cases} \equiv \text{circ}[\frac{L}{w}] \equiv \text{circ}[\frac{2w}{w}] \) (4.6)
4.1 Resolution of Optical Microscopes

- So, \( F[H] = A \cdot \frac{J_1[2\pi W \rho]}{2\pi W \rho} = h \), where \( J_1 \) is a Bessel function of the 1st kind and order

- So the image of a “point” becomes:

\[
|h[x, y]|^2 = A^2 \cdot \left| \frac{J_1[2\pi W \rho]}{2\pi W \rho} \right|^2
\]

(4.7)

- Since \( W_M = \frac{x_M}{\lambda f} \) and \( 2W_M \rho = 1.22 \)

\[
2 \frac{x_M}{\lambda f} \rho = 1.22
\]

- So, \( \rho = 0.61 \frac{\lambda f}{x_M} \) (eq. 4.8)

- A point will be imaged as a smeared spot of diameter \( \rho = 0.61 \frac{\lambda f}{x_M} \)

The Airy Function
4.1 Resolution of Optical Microscopes

- Imagine that we have two such points. Then the resolution is the minimum distance between the points that are separated, which is $\rho$.

$$\rho = \text{resolution}$$

- An objective lens that allows higher spatial frequencies (or angles) provides a higher resolution.
4.1 Resolution of Optical Microscopes

- Definition: \( \sin \theta_1 = \tan \theta_1 = \frac{x_M}{f} \)

\[
\sin \theta_1 = NA = \text{Numerical Aperture} \quad (4.10)
\]

- The resolution becomes \( \rho = 0.61 \frac{\lambda}{NA} \) but \( \rho = \frac{\lambda}{2NA} \) is good enough.

- Compare Ob₁ and Ob₂ above:

\[
\theta_1 < \theta_2, \quad x_{M1} < x_{M2} \implies NA_1 < NA_2 \implies \rho_1 > \rho_2 \quad (4.11)
\]

- So Ob₂ provides a better resolution.
4.1 Resolution of Optical Microscopes

- In general, objectives are made out of several lenses => complex systems

\[ f = \text{focal distance measured from principal plane} \]
\[ W = \text{working distance} = \text{distance from F to physical surface of lens} \]
Entrance Pupil = image of physical aperture
\[ \Rightarrow f \text{ and entrance pupil determine numerical aperture i.e. resolution} \]
4.1 Resolution of Optical Microscopes

- Note: if the objective lens is immersed in a medium for which \( n \neq 1 \), then

\[
NA' = n \cdot NA \quad \Rightarrow \quad \rho' = \frac{\rho}{n}
\]  

(4.12)

- This means that it is possible for immersed objective lenses to have a better resolution.
4.2 Contrast

- The final image consists of a distribution $I[x, y]$ which is the result of absorption, scattering/diffraction, etc.
- Contrast = a measure of the intensity fluctuations across the image. In general, the more contrast the better.

Low Contrast

High Contrast
4.2 Contrast

- Microscope image
- 2 regions of interest: A, B
- N is the background noise (in sample) (4.13)
- Contrast:
  \[ C_{AB} = \left| S_A - S_B \right|; \quad S_{A,B} = \text{signal A, B} \]
- Contrast to noise ratio:
  \[ CNR_{AB} = \frac{C_{AB}}{\sigma_N} = \frac{\left| S_A - S_B \right|}{\sigma_N} \]
  \[ \sigma_N = \text{standard deviation of noise}. \]
  \[ \sigma_N^2 = \sum_i (S_i - \bar{S})^2; \quad S_i = \text{signal in pixels} \]
4.2 Contrast

- While resolution is given by the instrument, the contrast is given by the instrument/sample combination.
- Most biological structures (i.e. cells) are very transparent so $I[x, y]$ is flat, which means there is low contrast.
- They can be assumed “phase objects”.
- Example of a phase object:

![Diagram showing wave front and imaging system with N=1.5, 100 nm, glass profile, and phase grating.](image.png)
4.2 Contrast

- No absorption so $I[x, y] = \text{constant} \implies \text{contrast} = 0$
- **BUT:** the wave front carries information about the sample
  \[
  E[x, y] = E_0 \cdot e^{i\phi[x, y]}
  \]  
  (4.14)
- This is the expression for the field in the vicinity of a phase object.
- **Bright Field** microscopy produces low contrast images of phase objects
4.2 Contrast

- There are several ways to enhance contrast:
  - Endogeneous Contrast
    - Dark field
    - Phase contrast
    - Schlerein
    - Quantitative phase microscopy
    - Confocal
    - Endogeneous florescence
  - Exogeneous Contrast Agents
    - Staining
    - Florescent tagging
    - More recently
      - Beads (dielectric and metallic)
      - Nano
      - Quantum Dots
### 4.3 Dark Field Microscopy

- Consider the low contrast image

- Typical low pass filtering = remove $\Delta C$

- Then take the inverse Fourier Transformation
4.3 Dark Field Microscopy

- Actual Microscope

- High frequency components are enhanced (e.g. edges)

- Without the sample → Dark Field
4.4 Schlerein Method

- Not used very often nowadays
- Enhances Contrast
- Phase objects can be rendered visible
- Edges are enhanced
- Relates to Hilbert Transform.
4.4 Schlieren Method

- Exercise: Show the following for a real signal $f(x)$

$$f(x) \xrightarrow{\text{Fourier}} F(g) \xrightarrow{\text{Cut } \frac{1}{2}} F_t(g) \xrightarrow{\text{Inverse Fourier}} f(x)$$

$$\tilde{f}(x) \in \mathbb{C} \quad \text{and} \quad \tilde{f}(x) = \frac{1}{2} f(x) + i \frac{P}{2\pi} \int \frac{f(x')}{x-x'} dx' \quad \text{Hilbert}$$

To the left: David Hilbert a German Mathematician, recognized as one of the most influential and universal mathematicians of the 19th and early 20th centuries.
4.5 Phase Contrast Microscopy

- Developed by Frits Zernike (1935) yielding Noble prize in 1953 (Physics)
- Very powerful, commonly used today.
- Consider a phase object:
  \[ U(x, y) = e^{i\phi(x, y)} \]  
  (4.15)
- Intensity distribution:
  \[ I(x, y) = |U|^2 \]
  \[ =1 \Rightarrow \text{No Contrast} \]
- Assume: The microscope has a magnification \( M=1 \)

![Diagram of phase contrast microscopy with Fourier Plane and Image plane]
4.5 Phase Contrast Microscopy

\[ \tilde{U}(f_x, f_y) = \int_\infty -\infty U(x, y)e^{-i2\pi(\xi f_x + \eta f_y)} \, dx \, dy \quad (4.16) \]

\[ f_x = \frac{x}{\lambda f} ; \quad f_y = \frac{y}{\lambda f} \]

- Note: \( \tilde{U}(0, 0) = \int_\infty -\infty U(x, y) \, dx \, dy \quad (4.17) \)
- Central Ordinate Theorem
- Zero Frequency component corresponds to a plane wave in the image plane (constant of \((x,y))\)}

\[ \int \int U(x, y) \, dx \, dy \quad U_0 = \frac{1}{A} \int \int U(x, y) \, dx \, dy \]
4.5 Phase Contrast Microscopy

- Note:
  - $U_0$ has no information about the structure of the sample.

\[
U_0 = \frac{1}{A} \int \int U(x, y) dx dy = \text{Average field}
\]

Image formation is an interference between the average field and high frequency components.

\[
U(x, y) = U_0 + [U(x, y) - U_0]
\]

(4.18)

High Frequency Component $U_1(x, y)$
4.5 Phase Contrast Microscopy

- Phase contrast relies on shifting the phase of $U_0$ by
  \[
  \frac{\lambda}{4} U_0 \rightarrow U_0 e^{i\alpha}
  \]
- Assume $|U_0| = 1$; $U_0 \rightarrow U_0 e^{i\alpha}$ becomes: $U(x, y) = ae^{i\alpha} + [U(x, y) - 1]$
- The intensity distribution in the image plane becomes:
  \[
  I(x, y) = |U(x, y)|^2 = \\
  = |ae^{i\alpha} + e^{i\phi(x,y)} - 1|^2 = \\
  = a^2 + 1 + 1 + \text{Re}[2ae^{i(\alpha+\phi)} - 2ae^{i\alpha} - 2e^{i\phi}] = \\
  = a^2 + 2[1 - a \cos \alpha - \cos \phi + a \cos(\alpha + \phi)]
  \]
4.5 Phase Contrast Microscopy

- Note: For $a = 0$ recover Dark Field Microscopy
- Assume “small” phase shift

$$\cos \phi \approx 1; \alpha = \pm \frac{\pi}{2}$$

$$I(x, y) = a^2 + 2a \cdot \sin \alpha \cdot \sin \phi$$

$$= a^2 + 2a \cdot \phi(x, y) \cdot \sin \alpha$$

$$I(x, y) = a^2 \pm 2a \cdot \phi(x, y) \quad (4.21)$$

- PC couples $\phi$ into intensity
- $a<1$ enhances contrast (best modulation for $|U_0| \approx |U_1|$)
4.6 Nomarski/Differential Interference Contrast Microscopy

- DIC = Differential Interference Contrast

- Use polarization discrimination to create 2 interfering beams
- Illuminate sample(s) with 2 drifted beams
4.6 Nomarski/Differential Interference Contrast Microscopy

- “Shift” amount ≈ Airy disk
  \[ \approx \frac{\lambda}{2NA} \]

- Wollaston prism #2 brings the 2 beams together through interference.

\[ E_{Total} = E_1 + E_0 = A_1 \cdot e^{i\phi_1} + A_0 \cdot e^{i\phi_0} \]

\[ \delta = \phi_1 - \phi_0 = n_1 \frac{d}{\cos \theta} k - n_0 \cdot d \cdot k \]
4.6 Nomarski/Differential Interference Contrast Microscopy

- By varying the position of Wollaston prism one can adjust $\delta = \phi_1 - \phi_0$
- Phase Shift through the sample:

\[
\begin{align*}
\text{dx} & \quad \text{l} & \quad \text{x} & \quad \text{e}^{i\phi(x+dx)} \\
. & \quad \text{s} & \quad \text{e}^{i\phi(x)}
\end{align*}
\]

- becomes:

\[
E_{Total} = A_n e^{i(\phi_n + \delta)} + A_0 \cdot e^{i\phi_0} = \approx A_0 e^{i\phi_0} \left[1 + e^{i(\phi_1 - \phi_0 + \delta)} \right] \quad (4.23)
\]
### 4.6 Nomarski/Differential Interference Contrast Microscopy

- The intensity in the image plane (as a function of displacement $x$).

\[
I(x) = 2I_0 (1 + \cos[\phi(x + dx) - \phi(x) + \delta])
\]

(4.24)

- Note: For small $\phi$, best results obtained for $\delta = \frac{\pi}{2}$

\[
I(x) = 2I_0 (1 + \sin[\phi(x + dx) - \phi(x)])
\]

\[
\approx 2I_0 [1 + dx \cdot \frac{\phi(x + dx) - \phi(x)}{dx}]
\]

(4.25)

- So the final intensity distribution is related to the gradient of the phase: $\frac{\delta \phi(x)}{\delta x}$
4.6 Nomarski/Differential Interference Contrast Microscopy

- DIC is a very sensitive to edges, even though the actual phase shifts are “small”.
- Example:

- Phase contrast and DIC heavily used today, especially for investigating live biological structures (cells) noninvasively.
4.7 Quantitative Phase Microscopy

- PC & DIC are great, but qualitative in terms of phase
- Knowing $\phi(x,y)$ quantitatively offers some advantages, i.e. gives a map of structure density; for homogeneous structures, gives molecular information.
- QPM is a “rather new” domain; several methods so far.
- Main obstacle is noise
4.8 Confocal Microscopy

- So far, we discussed full-field imaging, i.e. obtaining the entire image at once (great feature: imaging as a parallel process).
- The image can be recorded point by point also (like TV), sometimes with some advantages.
- Confocal = same focal point for illumination and collection
4.8 Confocal Microscopy

- Due to pinhole, light out of focus is rejected, which can create stacks of slices, hence 3D rendering
- Scanning: either by scanning the sample or the beam
- Note:
  - 3D Info
  - large field of view (limited by aperture)
  - up to $\sqrt{2}$ better resolution
- ! It works in reflection usually
4.8 Confocal Microscopy/ NSOM

- Recent development: Multi Foci
  - Improves acquisition time
  - Need more power → Trade-off

- Confocal can provide many frames/seconds (video rate)
- Leading to 4D imaging (x, y, z, t)
4.8 Confocal Microscopy/ NSOM

- Near Field Scanning Optical Microscope (NSOM)
  - Continuation of confocal & AFM
  - Tappered fiber as cantilever:

  ![Diagram](image)

  - Aperture down to 50 nm
  - Evanescent waves (no transmission in air)
4.8 Confocal Microscopy/ NSOM

Evanescent waves couple into sample → Became propagating
→ Not limited by diffraction

- Drawback: scanning time; difficult in liquids

$\delta = 50 - 100\,nm$
4.9 Fluorescence Microscopy

- Illumination and emission have different wavelengths
- Endogenous “Fluorophores” eg. NADH
- Most commonly exogenous
- Recently:
  - GFP technology (given fluorescent protein)
  - genetically encoded, fused with DNA
  - GFP live cell imaging
  - allows for multiple “fluorophores”
  - dynamic monitoring of processes(cell signaling)
4.9 Fluorescence Microscopy

- Fluorescence adds specificity to the measurement. (organelle dynamics, process specific)
- Typically- epi-fluorescence (reflection geometry)

- Filter blocks the excitation light
4.9 Fluorescence Microscopy

- Full-Field is limited to thin samples
- Combine fluorescence & confocal leads to deeper penetration
- Issues when imaging live cells:
  - acquisition time, sensitivity, damage.
- Photo-bleaching can produce cell damage:
  - limit duration of illumination \(\rightarrow\) need efficiency sensitivity
  - use intensified CCD
- Acquisition speed: improve with multi-foci & Nipkow disk scanning
4.9 Fluorescence Microscopy

- Other Fluorescence Techniques:
  - Total internal reflection
  - FCS-Fluorescence correlation spectroscopy
  - FRAP-Fluorescence recovery after photobleaching
  - FRET-Fluorescence resonance energy transfer.
  - FLIM-fluorescence lifetime imaging.
  - STED-Stimulated emission depletion → 100nm spot
  - STED+ 4Pi confocal microscopy 33nm diffraction spot
    single molecule imaging.
  - PALM, fPALM
  - Fiona, etc
4.10 Multiphoton Imaging

- 2-Photon laser scanning microscopy
- Nonlinear process
- Deep Penetration
- Requires high power density

\[ I_f \sim I_x^2 \] Improves Resolution

Fluorescence

Illumination spot is reduce by \( \frac{1}{\sqrt{2}} \)
4.10 Multiphoton Imaging

- 2nd harmonic Imaging-recent:
- Endogenous SHG molecules (e.g. collagen)
- $P = \chi^{(2)} \cdot E^2$ coherent process (phase matching)
- Same advantage of smaller illumination spot

Let’s take a look at examples!
Microscopy images

http://www.microscopyu.com/
Nikon
**Buccal Epithelial Cells - Phase Contrast**
Squamous epithelial cells divide about once in every 24-hour period and line the human mouth as part of the buccal muscosa. The cells secrete mucus, the substance that is the principal component of mucus. With the additional help of the salivary glands, the mucin keeps the mouth's interior moist. The mouth needs to maintain moisture in order to help secreted enzymes soften food, facilitate swallowing and begin the digestion process.

**Buccal Epithelial Cells - DIC**
The nucleus of each tiny cheek cell holds the genetic make-up of the entire body. Consequently, cheek cells are frequently used in diagnostic tests, such as paternity determination, and DNA fingerprinting efforts. A group of scientists in Australia, however, have found a new use for cheek cells. The researchers are examining the sodium transport level of squamous epithelial cells in children to determine whether or not they have a predisposition for developing hypertension. If their studies and testing procedures prove successful, those with a greater propensity may receive early warning and will be able to take preventative measures to avoid the condition.
1. Cell imaging

**HeLa Cell Culture - Phase Contrast**

The woman from which the HeLa cells are derived was named Henrietta Lacks, and she was a wife and mother of five when she passed away at John Hopkins University at the age of thirty-one. A sample of one of her tumors was sent to George and Maragret Gey who had been seeking a line of human cells that would survive indefinitely outside the body for research purposes. The tumor cells they received multiplied like nothing they had ever seen before and soon the cells, dubbed HeLa in a truncated form of Lacks' name, were being shipped to their colleagues stationed around the world. The cells later became a laboratory standard and have even been grown in space.

**HeLa Cell Culture - DIC**

Interestingly, the aggressive nature of HeLa cells in culture led not only to scientific discoveries, but also to experimental mistakes. HeLa cells can easily invade other cultures during routine lab transfer procedures and had contaminated many other cell lines used in research by the late 1960s. As a result, numerous research papers that were published based on a variety of cultured cell types have subsequently been proven to be HeLa cells and have, therefore, been discounted.
Fluorescence microscopy

http://www.microscopyu.com/
Nikon
Normal African Green Monkey Kidney Fibroblast Cells (CV-1 Line)

The intracellular relationship between the cytoskeletal filamentous actin network and mitochondria present in a culture of CV-1 fibroblast cells (illustrated above) was visualized with the use of the probes Alexa Fluor 488 conjugated to phalloidin (yielding green fluorescence emission) and MitoTracker Red CMXRos. Cell nuclei were counterstained with DAPI (blue emission). Images were recorded in grayscale with a 12-bit digital camera coupled to either a Nikon E-600 or Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Human Brain Glioma Cells (U-118 MG Line)

The U-118 MG glioma cells presented in the digital image above were resident in a culture that was immunofluorescently labeled with anti-tubulin mouse monoclonal primary antibodies followed by goat anti-mouse Fab fragments conjugated to Texas Red. In addition, the specimen was stained with Alexa Fluor 488 conjugated to phalloidin and Hoechst 33342, targeting the cytoskeletal filamentous actin network and UNA in the cell nucleus, respectively. Images were recorded in grayscale with a 12-bit digital camera coupled to either a Nikon E-600 or Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Human Cervical Adenocarcinoma Cells (HeLa Line)

A log phase culture of HeLa cells was transfected with three chimeric plasmid subcellular localization vectors (DsRed2-Mitochondria, ECFP-Golgi Complex, and EYFP-Nucleus), thus localizing a red protein tag to the intracellular mitochondrial network, a cyan tag to Golgi bodies, and a yellow tag to cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to either a Nikon E-600 or Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Human Cervical Adenocarcinoma Cells (HeLa Line)
The adherent culture of human carcinoma cells presented in the digital image above was transfected with a triplet of chimeric plasmid subcellular localization vectors. DsRed2, ECFP, and EYFP plasmid vectors were utilized to localize the nucleus, mitochondria, and actin, respectively. Imagae were recorded in grayscale with a 12 bit digital camera coupled to either a Nikon E-600 or Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Human Cervical Adenocarcinoma Cells (HeLa Line)

Transient transfection of a log phase culture of HeLa cells (illustrated above) with multiple chimeric plasmid subcellular localization vectors (DsRed2-Nucleus, ECFP-Mitochondria, and EYFP-Tubulin) enabled the localization of a red protein tag to the cell nuclei, a cyan tag to the intracellular network of mitochondria, and a yellow tag to microtubules. Images were recorded in grayscale with a 12-bit digital camera coupled to either a Nikon E-600 or Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
**Human Cervical Adenocarcinoma Cells (HeLa Line)**

Nuclei and the intracellular mitochondria and microtubule networks were localized in the HeLa carcinoma cell culture presented in the digital image above, which was transiently transfected with a chimeric ECFP plasmid vector that expresses a fluorescent fusion protein targeted at mitochondria and an EYFP plasmid vector that expresses a protein targeted at tubulin. The cells were also transfected with a recombinant plasmid vector containing a chimeric fusion gene product of DsRed2 fluorescent protein and a nucleus targeting sequence. Images were recorded in grayscale with a 12-bit digital camera coupled to either a Nikon E-600 or Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Human Cervical Adenocarcinoma Cells (HeLa Line)

HeLa cells are virulent and grow rapidly, reproducing an entire generation about once every 24 hours. In the 1970s, it was discovered that HeLa cells had invaded many other cell lines, invalidating millions of dollars of research carried out on a different type of cell than previously thought. Testing has revealed human papilloma virus 18 (HPV-18) sequences contained in HeLa cells, which express low levels of the tumor suppressor protein p53. Levels of pRB, a retinoblastoma suppressor protein, have been found to be normal. Cellular products of HeLa cells include keratin and lysophosphatidylcholine.
Human Cortical Neuronal Cells (HCN-1A Line)

Immunofluorescence with mouse anti-\(\alpha\)-tubulin was employed to visualize distribution of the microtubule network in the human cortical neuronal cell culture illustrated above. The secondary antibody (goat anti-mouse IgG) was conjugated to Cy2 and mixed with Alexa Fluor 568 conjugated to phalloidin to simultaneously image tubulin and the actin cytoskeleton. Nuclei were counterstained with Hoechst 33258. Images were recorded in grayscale with a 12-bit digital camera coupled to either a Nikon E-600 or Eclispe 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
2. Tissue slice imaging

**Distribution of Myelin Basic Protein and Glial Fibrillary Acidic Protein in Rat Brain Tissue**

In order to visualize myelin sheaths and astrocyte in a rat brain sagittal tissue section (shown above), the specimen was immunofluorescently labeled with mouse anti-myelin BP and rabbit anti-GFAP primary antibodies followed by goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively. Hoechst 33342 was employed to counterstain cell nuclei. Images were recorded in grayscale with a 1.2-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Horizontal Section of Rat Brain Immunofluorescently Labeled for Phosphorylated Neurofilaments

A horizontal section of rat brain was immunofluorescently labeled for phosphorylated neurofilaments with mouse anti-NF-P antibodies followed by goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488. Neurofilaments are specialized intermediate filaments solely found in neurons, especially in their axons. In addition, glial fibrillary acidic protein, which is expressed in various astroglia and neural stem cells, was targeted with rabbit anti-GFAP antibodies visualized with goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 568. Cell nuclei were labeled with the popular nuclear counterstain Hoechst 33342. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Brain Tissue Sample Triple Labeled with Alexa Fluor 488, Alexa Fluor 568, and Hoechst 33342

Immunofluorescence was utilized to label neurofilaments and astrocytes in a thin section of rat brain tissue. First, the specimen was fixed, permeabilized, blocked with 10-percent normal goat serum, and treated with a cocktail of mouse anti-NF-P (phosphorylated neurofilaments) and rabbit anti-GFAP (glial fibrillary acidic protein) primary antibodies. Then, to visualize the primary targets, the tissue section was treated with goat anti-mouse and anti-rabbit secondary antibodies (IgG) conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively. Finally, Hoechst 33342 was employed to counterstain cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Distribution of Neurons and Glia in Neural Tissue

This widefield fluorescence image of a rat brain tissue section was produced by probing the specimen with Alexa Fluor 488, Alexa Fluor 568, and Hoechst 33342. The two Alexa Fluor dyes were conjugated to secondary antibodies directed against primary mouse anti-NF-P antibodies and rabbit anti-GFAP antibodies in order to label phosphorylated neurofilaments expressed in neurons (Alexa Fluor 400) and glial fibrillary acidic protein in astrocytes and certain other astroglias (Alexa Fluor 568). The nuclear counterstain Hoechst 33342 was employed to visualize cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Glial Fibrillary Acidic Protein and Heavy Chain Neurofilament Subunits in Brain Tissue

Immunofluorescence was utilized to label neurofilaments and astrocytes in a horizontal section of rat brain tissue. First, the specimen was fixed, permeabilized, blocked with 10-percent normal goat serum, and treated with a cocktail of mouse anti-NF-H Non PO4 and rabbit anti-GFAP primary antibodies. Then, to visualize the primary targets, the tissue section was treated with goat anti-mouse and anti-rabbit secondary antibodies (IgG) conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively. Finally, Hoechst 33342 was employed to counterstain cell nuclei. Nuclear DNA was counterstained with Hoechst 33342. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Neural Tissue Labeled for GFAP, Myelin BP, and DNA

Myelin basic protein, which is a marker for the fatty sheaths surrounding the axons of myelinated nerve fibers, and glial fibrillary acidic protein, a type III intermediate filament protein found primarily in astroglia, were immunofluorescently labeled in the rat brain sagittal tissue section presented above by treating the specimen with a cocktail of mouse anti-myelin BP and rabbit anti-GFAP primary antibodies followed by goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively. Hoechst 33342, a dsDNA-interactive agent, was utilized to target cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
**Strata of the Rat Brain Visualized with Double Immunofluorescence and Synthetic Fluorophores**

In order to visualize myelin sheaths and astroglia in a rat brain sagittal tissue section (illustrated above), the specimen was immunofluorescently labeled with mouse anti-myelin BP and rabbit anti-SFAP primary antibodies followed by goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively. Hoechst 33342 was employed to counterstain cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Distribution of Neurofilament Protein and Glial Fibrillary Acidic Protein in Samples of Rat Brain Tissue

This widefield fluorescence image of a rat brain tissue section was produced by probing the specimen with Alexa Fluor 488, Alexa Fluor 568, and Hoechst 33342. The two Alexa Fluor dyes were conjugated to secondary antibodies directed against primary mouse anti-NF-P antibodies and rabbit anti-GFAP antibodies in order to label phosphorylated neurofilaments expressed in neurons (Alexa Fluor 488) and glial fibrillary acidic protein in astrocytes and certain other astroglia (Alexa Fluor 568). The nuclear counterstain Hoechst 33342 was employed to visualize cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles.
Rat Brain Tissue Immunofluorescently Labeled for Myelin Binding Protein and GFAP

Myelin basic protein, which is expressed in the fatty sheaths surrounding the axons of myelinated nerve fibers and glial fibrillary acidic protein, a type III intermediate filament protein found primarily in astroglia, were immunofluorescently labeled in the rat brain sagittal tissue section presented above by treating the specimen with a cocktail of mouse anti-myelin BP and rabbit anti-GFAP primary antibodies followed by goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively. Hoechst 33342, a dsDNA-interactive agent, was utilized to target cell nuclei. Images were recorded in grayscale with a 12-bit digital camera coupled to a Nikon Eclipse 80i microscope equipped with bandpass emission fluorescence filter optical blocks. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles, with the exception of Alexa Fluor 568, which was pseudocolored blue, and Hoechst 33342, which was pseudocolored red.
Confocal fluorescence

A common form of skin cancer (90 percent of the cases), basal cell carcinoma may grow slowly at first, but like other malignant cancer cells, can spread to other parts of the body (metastasize). It is estimated that 500,000 new cases of this type of non-melanoma skin cancer occur in the United States each year with about 2,000 deaths annually.