Advanced light microscopy

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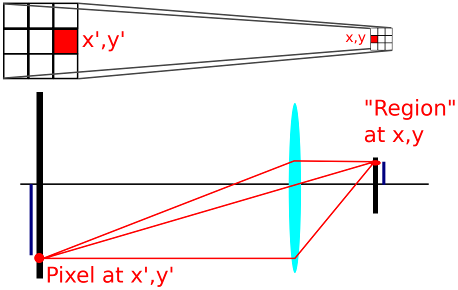
Literature

- OTFs and co.:
  Introduction to Fourier optics
  Goodman, Joseph W.
  maybe take the 2005 3. edition, not 1968
- Light sources and microscopy design:
  Zeiss campus website
  http://zeiss-campus.magnet.fsu.edu

Recapitulation: Magnification / Pixels

$$M(x, y) = I \cdot S(x, y)$$

- Sample $S(x, y)$ reacts to illumination I, measured as $M(x, y)$.
- **Magnification** links rectangular pixels ($d = 50 \ldots 150\,\mu\text{m}$) to areas on the samples focal plane (e.g. $d' = 75\,\mu\text{m}$ and $f = 60\times$ to $d = 125\,\text{nm}$).
- Think *effective pixel size*.

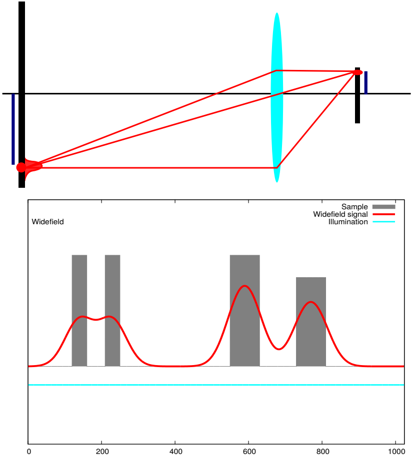


Camera pixel: Rectangular area collecting photons, thus integrating intensity. Maps to a (usually and ideally) regtangular area on the sample.

Recapitulation: Point-spread functions

$$M(x, y) = \mathrm{PSF} * (I \cdot S(x, y))$$

- **Point-spread function (PSF)** describes intensity distribution of point-like emitter.
- Measurement given by the sample intensity distribution folded by the PSF.
- Folding: Multiplication in Fourier space. Fourier transformation of the PSF: **Optical transfer function (OTF)**.



More detail: Ideal PSFs/OTFs and approximation

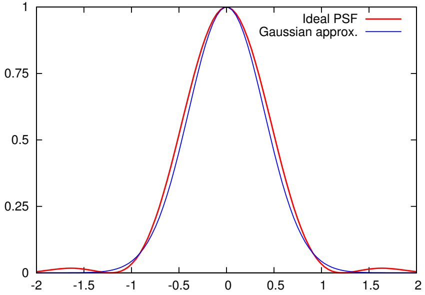
$$\tilde{M}(k_x, k_y) = \mathrm{OTF}(k_x, k_y) \cdot \left(I\tilde{S}(k_x, k_y) \right)$$

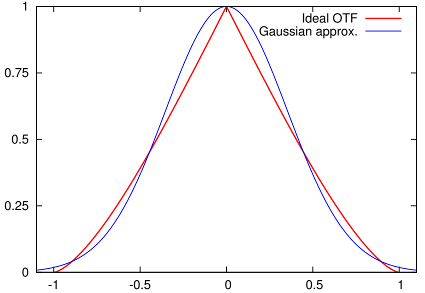
Ideal means a circular aperture in the Fourier plane, otherwise perfect optical system. Then:

$$\mathrm{PSF}(x) = \frac{2J_1(x)}{x}^2$$

$$\mathrm{OTF}(k) = \frac{2}{\pi} \left(\arccos(|k|) - |k|\sqrt{1-k^2} \right)$$

Gaussian approximation not too bad for PSF, and Fourier transform gets much easier.



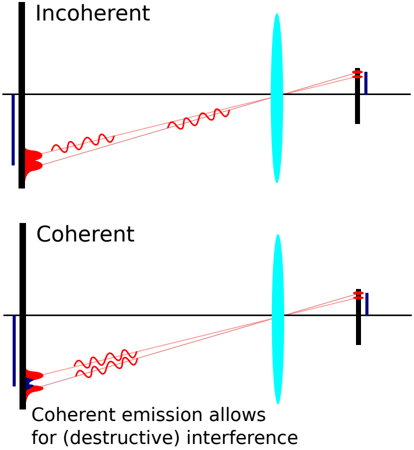


Recapitulation: Coherence

- This

$$M(x,y) = \mathrm{PSF} * (I \cdot S(x,y))$$

  implies adding up intensities. Or does it? Complex valued I, S, PSF...
- **Coherence**: Does the sample preserve the phase of incoming light?
- **Fluorescence** lifetime in order of nanoseconds, destroys coherence. For other materials and processes, a closer look might be needed (e.g., stimulated coherent emission).



What is **Background**, what is **Noise**

What is background, what is noise

Noise is an uncertainty in measuring the number of photons on a pixel. **Background** are photons picked up from (usually out-of-focus) positions on the sample that are of no interest to the measurement.

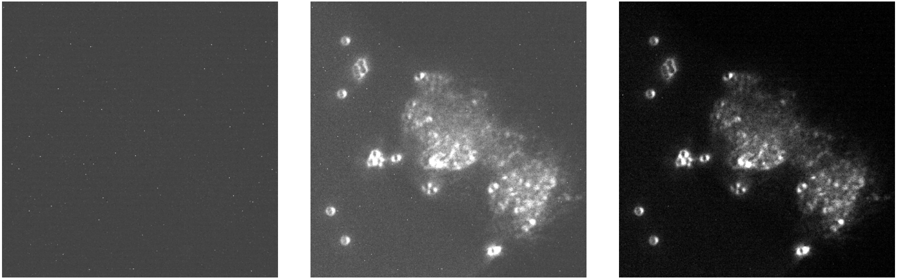
In common

- Both add to the measurement.
- Both are **not signal**. Thats somewhat by definition, as in *not the signal we are interested in*.
- Sometimes low enough to ignore, sometimes so high the image is ruined.
- Some post-processing addresses both.

Differences

- Noise is a camera property, while background depends on
  - the sample
  - the way it is illuminated
  - the way it is imaged
- Often, with $M = I \cdot S$, increasing I keeps noise constant, while background scales with I. In these cases: **More light improves SNR, but not SBR**.

Noise



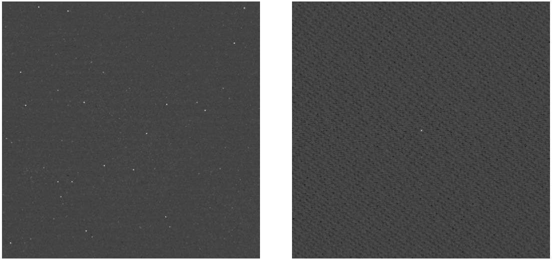
Noise/dark frame (left), full signal (middle), signal with darkframe substraction (right) [1]

- **Noise** arises as the camera (mis)counts the number of photons.
- Photo effect: **Thermal and quantum effects** distort the number of electrons.
- **Thermal and quantum effects** distort the amplification and conversion from electron charge to a digital value.

- **More light** (at least for SNR, see last slide)
- **Cooling** helps with some of the thermal effects.
- **Dark frame substration** (below)
- **"Better" electronics** helps, lecture on camera types and trade-offs (noise, speed, price).

[1] Images provided by Christian Pilger

Noise in Fourier space

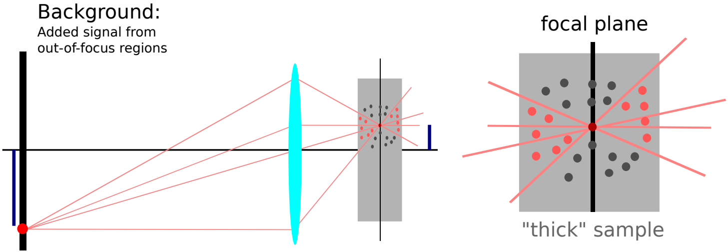


Noise in spatial (left) and frequency (right) domain

$$M(x,y) = (\mathrm{PSF} * (I \cdot S(x,y))) + N(x,y)$$

- $N(x,y)$ is random: Distribution influenced by various factors.
- Each pixels might be different, e.g. different average and variation.
- In first approximation there should be no correlation between pixels. Spectral distribution can be used to check.
- **High-frequency filtering** thus will eliminate some of it.
- **Camera characteristics** can be measured quite extensively.

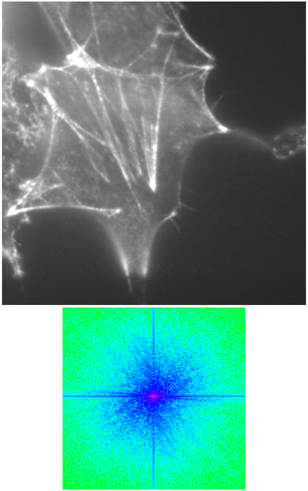
Background



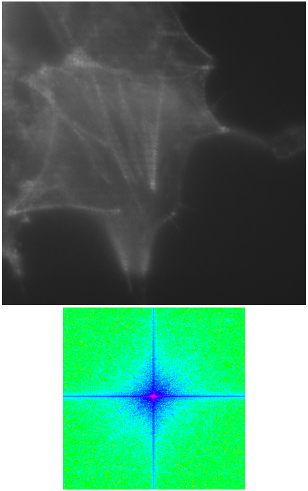
- Samples are often not flat, but somewhat thick.
- Image plane (photons stopped by camera chip) sets a fixed sample focal plane
- Pixels collect additional light from **out-of-focus contributions**

Background: Examples

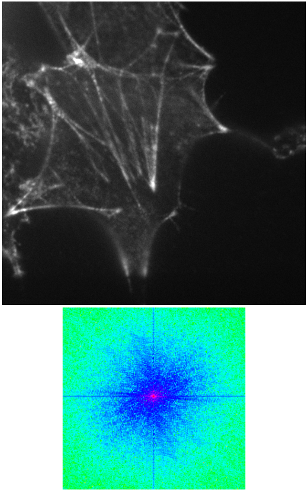
Full Image



Background



Full Image - Background

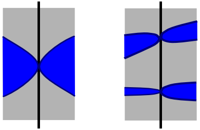


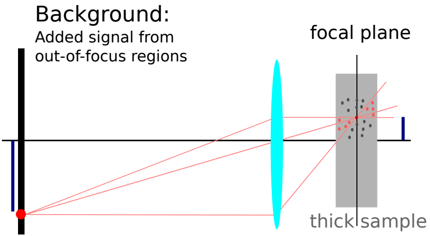
Background: 3D PSF

$$M(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I \cdot S(x,y,z))\,\mathrm{d}z$$

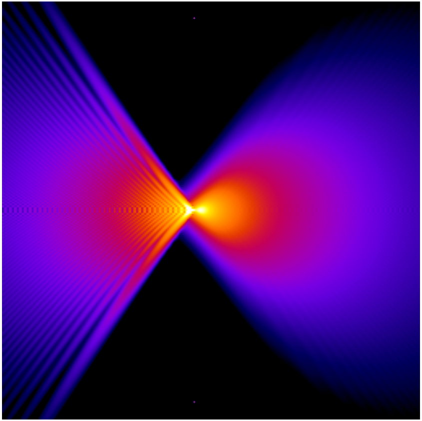
- 3D PSF (and sample) to account for these contribution
- z-component generally harder to calculate, but can be measured and/or simulated.
- **Important**: Axial vs. lateral resolution and improvement

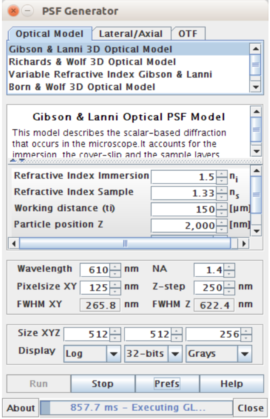
Sketch of 3D PSF





Background: Obtaining a PSF



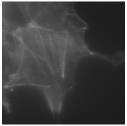


ImageJ Plugin: PSF Generator

- Simulation: Software takes into account particle position, working distance, all refractive index changes between immersion medium, cover slip, sample.
- Measurement: Use single point-like emitters (reasonably smaller than resolution limit) and scan them through the focus.

Summary: Background and Noise

$$M(x,y) = \underbrace{\int_{S_z} \text{PSF}(z)}\, * (I \cdot S(x,y,z))\,\mathrm{d}z + \underbrace{N(x,y)}$$



Background

- Unwanted out-of-focus contributions
- Background scales with illumination intensity, thus SBR (signal-background-ratio) uninfluenced by more light
- Improvements: Illumination, Deconvolution, . . .

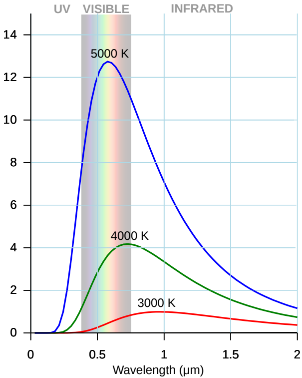
Noise

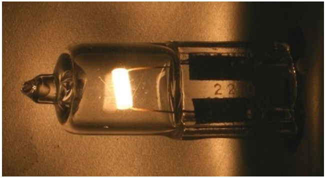
- Measurement errors for camera photon count
- Noise is constant when illumination is increased, thus SNR (signal-noise-ratio) can be improved by more light
- Other improvements: Exposure time, camera type (with cost/speed/noise trade-off).

Light and Illumination: Sources, Modes of Illumination

Light sources: Incandescent light bulb

- Patent 1845, Osram halogen in 1959
- Heated tungsten, approx. black body spectrum, halogen for higher temperature
- Light: No coherence, not at all monochrome
- Filtering to a narrow-band emission throws away lots of the spectrum
- Historically cheap and easy source, today rivaled by (cheap) high power LEDs

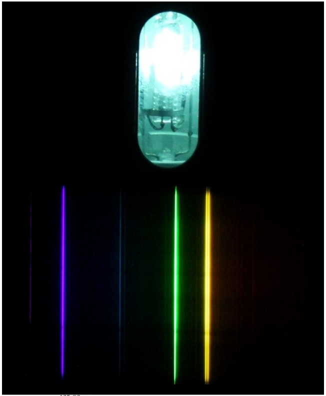


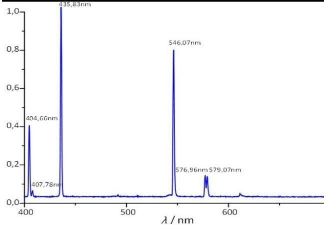


Wikimedia/Spektrum, Wikimedia/Halogen Lamp

Light sources: (mercury) gas-discharge lamp

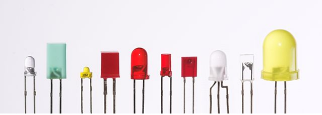
- Mercury lamps: discovered 1705, first applications 1901, referred to as *burners*
- Light generated by electrical discharge that ionizes gas
- No coherence, spectral lines given by gas, spectral broadening when using high pressure
- Filtering to narrow-band emission is quite effective if close to spectral line.
- Today still in use, again rivaled by (quality) high power LEDs and Lasers.





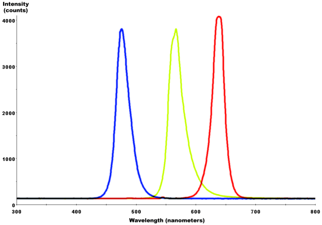
Wikimedia/Hg-Spektrum

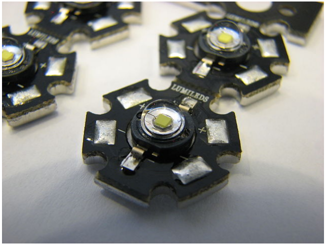
Light sources: High Power LEDs (Light-emitting diode)



Wikimedia/LEDs

- Prototype 1962, blue LEDs 1994, Nobel Prize 2014
- Semiconductor diode with band gap photon emission
- Light: No coherence, wave length set by band gap, spectral peak somewhat broad (compared to lasers)
- Filtering gives a narrower band if required
- High power LEDs became available only some years ago
- Today: Go-to light source (that is cheaper than a laser)





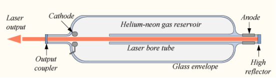
Wikimedia/LED-Spectrum

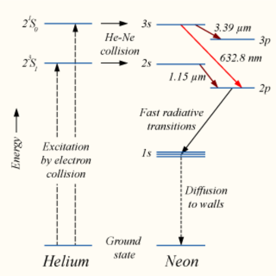
Wikimedia/High Power LED

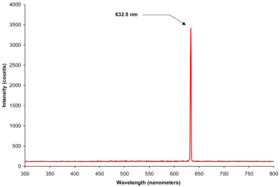
Light sources: LASER / principal (light amplification by stimulated emission of radiation)

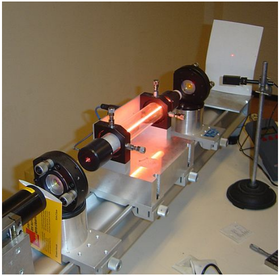
- Patent 1960, semiconductor laser diodes since approx. 1990
- Laser principle: Stimulated emission in a system with multiple energy states
- Different types, most important:
  - Gas (e.g. He/Ne)
  - solid state/crystals
  - semi-conductor/diode

Also, pumping one type (crystal) with another (diode).







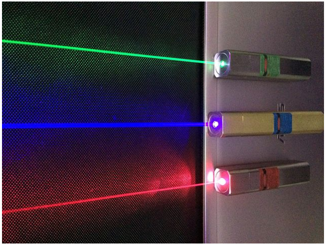


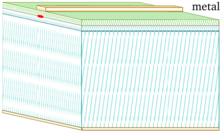
Wikimedia/HeNe diagram
Wikimedia/HeNe states
Wikimedia/HeNe spectrum

Wikimedia/HeNe laser

Light sources: LASER / application

- Laser diodes: Similar to LEDs, but withstand higher local currents and (might) have a more complex band structure.
- Laser light (in general): Very coherent, single wave length, narrow band
- Filtering still useful, especially for semi-conductor lasers
- **Coherent light** is very versatile when designing optical systems: Think gratings, interference patterns.
- Today: Generally laser diodes (cheap, powerful), other types for special requirements (good coherence, multiple spectral lines).





Wikimedia/Lasers

Wikimedia/Laser diode

Spectrum of LEDs and Laser diodes

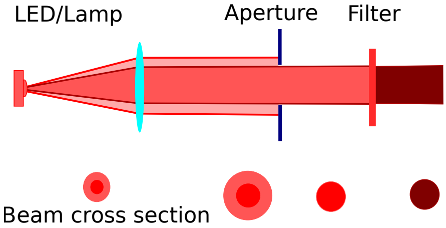
Color	Wavelength [nm]	Voltage drop [ΔV]	Semiconductor material
Infrared	$\lambda > 760$	$\Delta V < 1.63$	Gallium arsenide (GaAs) Aluminium gallium arsenide (AlGaAs)
Red	$610 < \lambda < 760$	$1.63 < \Delta V < 2.03$	Aluminium gallium arsenide (AlGaAs) Gallium arsenide phosphide (GaAsP) Aluminium gallium indium phosphide (AlGaInP) Gallium(III) phosphide (GaP)
Orange	$590 < \lambda < 610$	$2.03 < \Delta V < 2.10$	Gallium arsenide phosphide (GaAsP) Aluminium gallium indium phosphide (AlGaInP) Gallium(III) phosphide (GaP)
Yellow	$570 < \lambda < 590$	$2.10 < \Delta V < 2.18$	Gallium arsenide phosphide (GaAsP) Aluminium gallium indium phosphide (AlGaInP) Gallium(III) phosphide (GaP)
Green	$500 < \lambda < 570$	1.9[70] $< \Delta V < 4.0$	**Traditional green:** Gallium(III) phosphide (GaP) Aluminium gallium indium phosphide (AlGaInP) Aluminium gallium phosphide (AlGaP) **Pure green:** Indium gallium nitride (InGaN) / Gallium(III) nitride (GaN)
Blue	$450 < \lambda < 500$	$2.48 < \Delta V < 3.7$	Zinc selenide (ZnSe) Indium gallium nitride (InGaN) Silicon carbide (SiC) as substrate Silicon (Si) as substrate—under development
Violet	$400 < \lambda < 450$	$2.76 < \Delta V < 4.0$	Indium gallium nitride (InGaN)
Purple	Multiple types	$2.48 < \Delta V < 3.7$	Dual blue/red LEDs, blue with red phosphor, or white with purple plastic
Ultraviolet	$\lambda < 400$	$3.1 < \Delta V < 4.4$	Diamond (235 nm)[71] Boron nitride (215 nm)[72][73] Aluminium nitride (AlN) (210 nm)[74] Aluminium gallium nitride (AlGaN) Aluminium gallium indium nitride (AlGaInN)—down to 210 nm[75]
Pink	Multiple types	$\Delta V \sim 3.3$[76]	Blue with one or two phosphor layers: yellow with red, orange or pink phosphor added afterwards, or white phosphors with pink pigment or dye over top.[77]
White	Broad spectrum	$\Delta V = 3.5$	Blue/UV diode with yellow phosphor

- **375 nm** – excitation of Hoechst stain, Calcium Blue, and other fluorescent dyes in fluorescence microscopy
- **405 nm** – InGaN blue-violet laser, in Blu-ray Disc and HD DVD drives
- **445 nm** – InGaN deep blue laser multimode diode recently introduced (2010) for use in mercury free high brightness data projectors
- **473 nm** – sky blue laser pointers, still very expensive, output of DPSS systems
- **485 nm** – excitation of GFP and other fluorescent dyes
- **510 nm** – (to ~525 nm) green diodes recently (2010) developed by Nichia and OSRAM for laser projectors.
- **635 nm** – AlGaInP better red laser pointers, same power subjectively twice as bright as 650 nm
- **640 nm** – high-brightness red DPSS laser pointers
- **650 nm** – GaInP/AlGaInP CDDVD, cheap red laser pointers
- **670 nm** – AlGaInP bar code readers, first diode laser pointers (now obsolete, replaced by brighter 650 nm and 671 nm DPSS)
- **671 nm** – spectroscopy, DNA sequencing, high-power red DPSS laser pointers
- **760 nm** – AlGaInP gas sensing: O_2
- **785 nm** – GaAlAs Compact Disc drives
- **808 nm** – GaAlAs pumps in DPSS Nd:YAG lasers (e.g. in green laser pointers or as arrays in higher-powered lasers)

Wikipedias List of LED (left) and LASER (right) wavelength and materials.

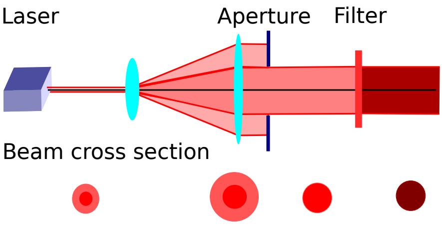
- Today: Variety of semiconductor materials to obtain different wavelength

Illumination/Source: Lamps and LEDs



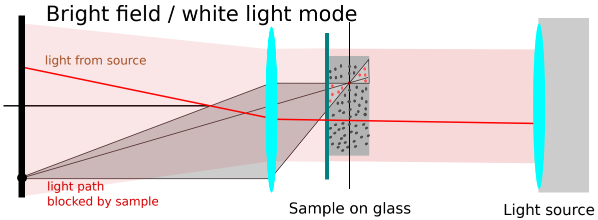
- Light emission under some angle
- Lens (system) to obtain a parallel beam
- Aperture to block out low intensity outer regions
- Filter to narrow spectrum (LED typ. 25 nm FWHM)
- Ideal result: A monochrome, parallel beam with uniform intensity

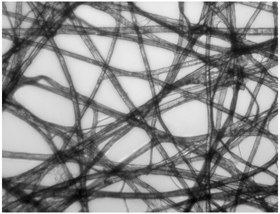
Illumination/Source: Lasers



- LASER emits almost parallel light
- Lens (system) to widen beam
- Aperture to block out low intensity outer regions. A nice profile is Gaussian, a diode can be much worse.
- Maybe: filter to clean up / correct spectrum. Native laser diode spectrum depends on technical details (current control, mechanical construction).
- Ideal result: A monochrome, coherent, parallel beam with uniform intensity

Illumination/"classic": Bright field / white light mode

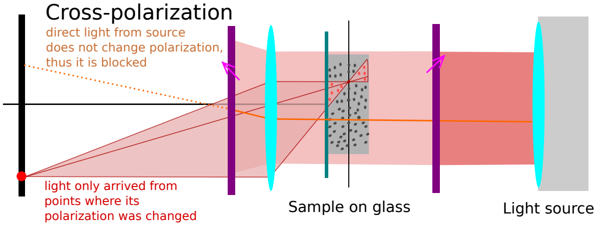


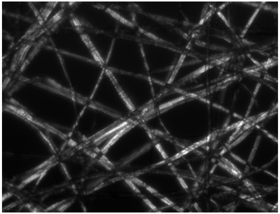


Wikimedia/Bright Field

- Arguably the oldest and most standard mode
- Light goes through the sample: "Durchlicht"
- Contrast by absorbing and scattering light
- Optimized by using **Köhler illumination**
- Used at (almost) every microscope e.g. to quickly align a new sample

Illumination/"classic": Cross-polarization

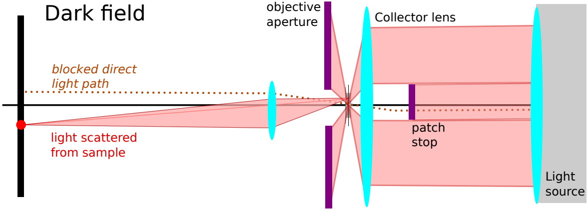


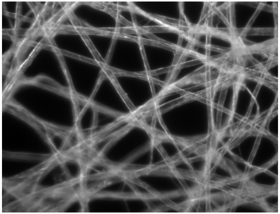


Wikimedia/Cross-polarized

- Easy extension to bright field: Two polarization filters
- Polarized light enters the sample:
  - If it does not interact, it is blocked by a second filter
  - If it interacts, and changes polarization, it can pass the second filter
- Visible: Structures that change the polarization of light

Illumination/"classic": Dark field

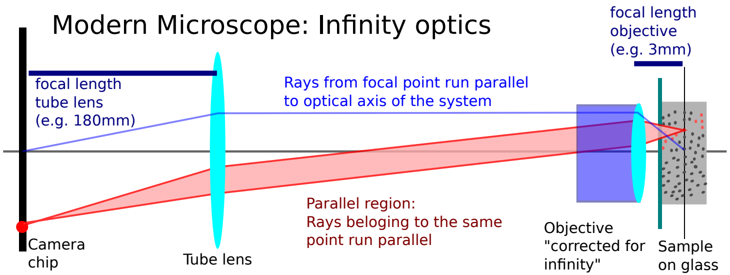




Wikimedia/Dark Field

- Align illumination optics and block central beams
- Light enters sample under a steep angle:
  - If it does not scatter, it exists under the same angle. Thus it is blocked by the imaging objectives aperture
  - If it scatters, some will leave under a flat angle, able to pass the aperture
- Visible: All scattering structures
- This can be extended to measure (almost only) phase-shifting structures.

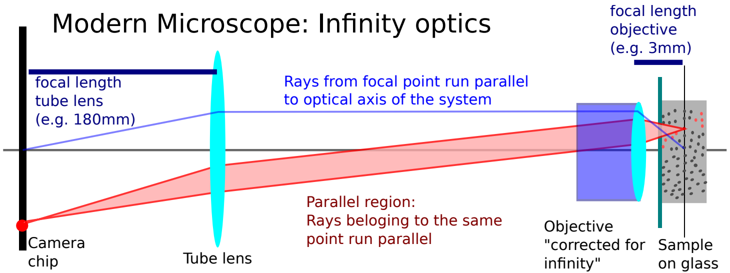
Excursion: Infinity optics



$$\frac{1}{f} = \frac{1}{b} + \frac{1}{g}$$

- Set $g = f$, then $b = \infty$, image forms "in infinity"
- Set $b = f$, then $g = \infty$, objects at infinity distance are now in focus
  That one actually works on it own, take a landscape photo with $g >> f$
- For an imaging system, plug two of these ($b_1 = f_1, g_1 = \infty$, $b_2 = \infty, g_2 = f_2$) together
- Advantage: "Parallel region" for light manipulation (filters, mirrors)

Excursion: Infinity objectives



- **Magnification** becomes easy: $\frac{f_1}{f_2}$, here $\frac{180\,\mathrm{mm}}{3\,\mathrm{mm}} = 60\times$
- "Infinity objectives" are no magical, physics-defying things
  - They are a bunch of (well made, well adjusted, expensive) lenses
  - "Infinity" means: Lens corrections (coating, calculations) are optimized for infinity focus applications (at more than one wavelength)
  - Often they state a magnification (60×), not a focal length (3 mm). In these cases, there is a (manufacturer-dependent) standard tube lens (e.g. 180 mm) that gives that magnification.