	optical microscopy					
	bright field	**dark field**	**phase contrast**	**polarization**	**DIC**	**FLuorescence in general**
Full Name	bright field microscopy	dark field microscopy	phase contrast microscopy	polarization microscopy	Differential Interference Contrast	Fluorescence
resolution	250 nm (theory) worse resolution due to blur	1.559 µm/pixel (apparantly 20 times better than bright field)	better than dark field (<1nm??)	not optimal	100 nm (z)	/
Static/Dynamic	Static & Dynamic	Static & Dynamic	Static & Dynamic	Static & Dynamic	Static & Dynamic	
timescale	real time	real time	real time	real time	real time	/
information	◉ unpolarized white light illumination ◉ imaging objects that absorb (visible) light ◉ often via staining ◉ high quality imaging by optimum illumination: Köhler illumination	◉ light scattering (density, fluctuations, small objects,...) ◉ illuminating the specimen at an oblique angle such that direct, nondiffracted rays are not collected by the objective. ◉ nondiffracted background light is absent from the image, light-diffracting objects look bright against a dark field	◉separation of light, which is diffracted by the object, from the light, which is not altered by the object, that is, the background or undiffracted light ◉amplitudes and phases of these two components are modified ◉ amplitude contrast occurs due to constructive or destructive interference between the two light components ◉making objects appear as if they had been optically stained	◉ compound light microscope fitted with a polarizer and an analyzer. The polarizer is placed between the light source and the specimen, commonly near the front aperture of the condenser; ◉ the analyzer is placed between the specimen and the eye, usually some distance behind the rear aperture of the objective. ◉ image formation is based on the existence of ordered molecular arrangements	◉views gradients in optical path lengths. ◉the image appear deceptively 3D and real ◉ no phase halos ◉ consists of polarizer, condenser DIC prism, objective DIC prism, analyzer	/
advantages	◉ Extremely easy ◉No sample preparation needed ◉Very common setup	◉ no staining needed in comparison to bright field ◉ no halos ◉ detect weak diffracted signals ◉ Relatively easy and cheap ◉ No undiffracted light = better contrast	◉ enhance the visibility of the light-scattering edges of extended objects and particles ◉ electronic enhancement and image processing ◉ objects containing just a few protein molecules can be detected.	◉Good for strucutrured samples	◉ high resolution ◉ high contrast	◉Better contrast than optical methods => fluorescence removes background by using stokes shift ◉It is easier for humans to distinguish fluo spot from a dark background than an optical image with lower intensity
disadvantages	◉ Low contrast (lot of non-diffracted background) ◉Not optimal resolution	◉ low light levels seen in the final image => needs intense light (can damage sample) ◉ lower sensitivity due to lack halos	◉ More difficult setup	◉ limited in resolution	◉High cost	/
examples	◉H&E staining ◉gram staining	most commonly used for minute light-diffracting specimens, such as diatoms, bacteria and bacterial flagella, isolated organelles and polymers, such as cilia, flagella, microtubules, and actin filaments, and silver grains and gold particles in histochemically labeled cells and tissues	see lecture 2	to study the form and dynamics of many ordered cellular structures, including: mitotic spindle fibers in dividing cells, actin filament bundles, actin and myosin filaments in the myofibrils of striated muscle cells, condensed DNA, helical strands of cellulose fibers in plant cell walls, condensates of starch and lignin in plant cells, virus crystalloids and crystals of organic compounds in the cytoplasm of plant cells, lipid bilayers of the cell plasma membrane and mitochondria.	see lecture 2	**/**
class	class 2 Optical Microsccopy	class 2 Optical Microsccopy	class 2 Optical Microsccopy	class 2 Optical Microsccopy	class 2 Optical Microsccopy	class 3 Fluorescence microscopy

Basic Fluorescence microscopy				Improved fluorescence microscopy	
wide field microscopy	**confocal microscopy**	**TIRF**	**two photon excitation**	**spinning disk microscopy**	**SPIM**
Wide field fluoresence microscopy, also called: epi-fluorescence microscopy	CLSM (Confocal Laser Scanning Microscopy)	Total Internal Reflection Fluorescence	Two photon excitation microscopy	also called NIPKOW disk	selective plane illumination or light-sheet microscopy
250 nm (xy) 800 nm (z)	150 nm (xy) 500 nm (z)		300 nm (xy) 1000 nm (z) due to double wavelengths of confocal	same as confocal	
Static & Dynamic	Static & Dynamic	Static & Dynamic	Static & Dynamic	Static & Dynamic	Static & Dynamic
> 10 fps	1-100 fps	1-100 fps	similar to confocal	1000fps	
◉uniform illumination of sample and area detector ◉fluorescence microscopes rely for efficient and high contrast imaging on the epi-illumination principle where, both illumination and detection are positioned on the same side of the microscope slide as the specimen. ◉Crucial are efficient fluorescence/color filter sets that contain three essential filters: excitation filter, dichromatic mirror and barrier/emission filter. ◉variety of light sources can be used going from lamps to lasers	◉ illuminating the specimen with a focused scanning laser beam (point scanning) and by placing a pinhole aperture in the image plane in front of an electronic photon detector (point detection). ◉ rejecting signals from nearby sources above and below the focal plane. ◉The confocal principle: Excitation wavelengths from a laser point source are confocal with a scanning point in the specimen. ◉ out-of-focus information is largely excluded from the detector and final image.	◉adapted form of widefield fluorescence microscopy that uses a specialized method of illumination to excite fluorescence in molecules located within less than 100 nm of the surface of the coverslip. ◉ illumination with evanescent wave --> excitation of moelcules < 100 nm inside sample	◉true 3D imaging ◉a form of laser-scanning microscopy with a pulsed near-infrared (IR) laser that excites fluorescence excitation by two-photon absorption and results in a highly localized excitation of fluorescence. ◉two photons of half the energy can excite a fluorophore --> excited state can fluoresce. ◉probability of simultaneous absorption of 2 photons is low (squared intensity) --> high flux of excitation photons is required= pulsed laser. ◉ multiphoton excitation allows deeper imaging up to 500 µm: 1) long wavelengths penetrate better, less Rayleigh scattering 2) lower absorbance of tissue ◉Non-linear technique	Confocal Imaging with a Spinning Disk: Instead of illuminating the object by raster scanning using a single spot, it is possible to scan the specimen with thousands of points simultaneously using a spinning Nipkow disk. A Nipkow disk contains thousands of minute pinholes arranged in multiple nested rows of outwardly spiraling tracks	◉illuminated with light-sheets to achieve optical sectioning: orthogonal-plane fluorescence optical sectioning (OPFOS) ◉The dimensions of the light-sheet can be adapted to different sample sizes: for smaller samples (20-100 µm), the light-sheet can be made very thin (~1 µm); whereas for larger samples (1-5 mm), the sheet has to be thicker (~5-10 µm) to remain relatively uniform across the field of view
◉easy to use ◉ low cost ◉ objective, first servings as well corrected condensor and then as the imageforming light gatherer, is always in corrrect alignment relative to each of these functions ◉most of the unwanted or unused excitation light reaching the specimen travels away from objective ◉ the area being illuminated is restricted to the area being observed ◉ the full numerical aperture of the objective is utilized ◉ it is possible to combine with transmitted light observation	◉optical sectioning through fluorescent objects up to 10- to 50-µm thick ◉ images with an improved contrast and Z-resolution (sqrt2) compared to wide field ◉ fine structural details visible	◉very high contrast and S/N ratio ◉Photodamage is also reduced and cell viability is prolonged	◉ low scattering (NIR) ◉excellent penetration (NIR) ◉ reduced bleaching (only at spot) ◉ decrease fluorescence background ◉ produce bright, high contrast, fluorescence images even at great depths in thick specimens ◉less biological damage when using NIR	◉ imaging performance good overall ◉ microlens transmission range 405 - 650 nm ◉Faster imaging than confocal	◉ higher local intensities ◉ faster acquisition ◉ less photobleaching
◉ no optical sectioning ◉Blur by fluorescence of different planes together!	◉fast physical movement of the specimen can cause wobble and distortion --> loss of resolution in the image. ◉impossible to perform various manipulations on the specimen such as microinjection of fluorescently labeled probes when the stage and specimen are moving. ◉ slow scanning ◉ low penetration	◉Only info close to glass coverslip	◉ slow scanning ◉ low resolution ◉ high high cost power beam	◉20,000 pinholes are spaced closely together on the disk, there is some crosstalk between neighboring pinholes, because the fluorescent light emitted from one site enters several pinholes on the disk, not just one pinhole ◉crosstalk adds to the background signal (background haze) --> reducing the S/N ratio of the object. ◉ pinholes in the disk are fixed in physical size and not variable. Thus, imaging may be somewhat compromised in resolution and z-section thickness for certain objectives	uneven light sheet thickness
◉screening ◉ thin sections ◉highlight the overall shape of the pollen grain and the outline of the nuclei in the brain section, the images are blurred by emission originating away from the focal plane.	◉medium-thick sections ◉surface of embryos		deep penetration in scattering tissue		imaging in millimeter sized embryos of medaka and Drosophila melanogaster
class 3 Fluoresence microscopy	class 3 Fluoresence microscopy	class 3 Fluoresence microscopy	class 3 Fluoresence microscopy & 11 labelfree imaging	class 3 Fluoresence microscopy	class 3 Fluoresence microscopy

	single molecule microscopy	FRET				
spectral detection	**single molecule microscopy**	**FRET A/D emission ratio**	**Acceptor Photobleaching FRET**	**FRET-FLIM**	**smFRET (Single molecule FRET)**	**NALM**
/	consists of scanning confocal fluorescence microscopy, wide-field microscopy, FCS, burst integrated FLIM, TIRF, scanning near-fiel optical microscopy, AFM, STM, FRET...	Förster resonance energy transfer				NAnometer Localization Microscopy
		0-10 nm (Förster Radius) - Spatial resolution is same as microscope you use				superresolution (few nm)
	dynamic & static	Dynamic				
		Depends on microscope used				minutes
⦿emission can be split in 2 (3) colors ⦿overlap of emission spectra between 2 (+) fluorescence markers (fluorescence cross talk/bleed through)	pinpoint location of individual emitter provided that you look at individual emitter	⦿Donor fluorophore gives absorbed photon energy to acceptor fluorophore that emits photon if they are close together ⦿ D and A must have compatible spectra ⦿Kind of superresolution (not really) ⦿Possible on every type of fluorescence microscope				Used for particles with multiple fluorophores. In time these fluorophores are bleached. Intensity decrease in steps. Subtraction of images of different number of active fluorophores ⦿ Widefield microscope ⦿sensitive and flexible
		⦿ Very easy (1 laser needed) ⦿ FRET in realtime ⦿ High FRET = high signal	⦿ Simple set-up (1 laser + 1 detector) ⦿ Measure FRET and no-FRET (after bleaching) on the same sample ⦿ More FRET = higher signal	⦿Measure Donor lifetime and FRET ⦿Acceptor does not need to fluores (can also be quencher!) ⦿Single channel since we only need to measure donor lifetime	⦿Done in confocal ⦿Can measure structural dynamics of molecules	
⦿ intrinsic dye properties (absorption & emission overlap) ⦿ optical filters: high cut-on/off band pass emission filters ⦿solution for both: sequential imaging		⦿ Two channel detection ⦿ Not that quantative => no R information	⦿ Acceptor must not be photostable ⦿ Not realtime! ⦿ bleaching only once	⦿ Bit more complicated set-up!		⦿Still relative long acquisit of photons detected is l
	nucleus, membranes, enzymes, lipids	⦿See slide 45 lecture 10 (something with more FRET in cytosol than in nucleus)	⦿ Something with HIV-integrase (slide 48 lecture 10)	⦿ Mechanical force measurement in adhesions		Dendimers with 4 fluorophores. Find location of each fluorophore
class 3 Fluoresence microscopy		Class 10 lifetimes, FLIM, FRET	Class 10 lifetimes, FLIM, FRET	Class 10 lifetimes, FLIM, FRET	Class 10 lifetimes, FLIM, FRET	class 8 superresolution microscopy

Single Molecule Localization Microscopy (also TIRF)				REversible Saturable Optical Fluoresence Transitions (RESOLFT)	
PALM(=STORM)	**S-PALM**	**STORM (=PALM)**	**3D localization microscopy**	**STED**	**POSH**
Photoactivated localization microscopy	stroboscopic palm	Stochastic Optical Reconstruction Microscopy		stimulated emission depletion STED microscopy	Photoswitching High resolution microscopy
xy: 20-30 nm z: 800 nm	~15 nm	xy: 20-30 nm z: 800 nm (in the last ppt it says 100-150 nm)	400 nm	xy: 50 nm z: 500 nm	60 nm
static	dynamic	static		Static	Static
minutes	25 fps aquisition time: 2min	minutes	minutes to hours	ps or 1 fps	ms
◉Setup: Wide-Field+TIRF Principle: Blinking of Photoactivable FPs, reconstruction of a superresolved image after fitting a point-spread function and piling them up ◉ multi color PALM possible ◉ use PA FPs as dyes (like dronpa)	The stroboscopic effect is a phenomenon due to interrupted illumination of a moving object. When an oscillating body is seen in periodically interrupted light (a series of light flashes occurring at a definite rate) it appears different	◉Recording super resolved image based on blinking of single molecules. --> found out that blinking of 1 of 2 dyes was enhanced by presence of 2nd dye. 1 serves as electron trap and releases the e-, restores fluorescence and so on. Not linked to triplet state but to e- transfer temporarily trapping of e- that is captured, released again, fluorescence resumed ◉When you have enough labelling strands on dna --> possible to measure circular structures ◉ use ALEXA647 dye ◉ multi color STORM possible	◉addition cylindrical lens in beam path --> round spot = molecule. if molecule is slightly out of focal plane --> elongated feature along x-axis. Below focal plane --> elongated feature along Y-axis Calibration ---> molecule is position above/below focal plane with x nm (max 400 nm range) ◉ color coded images ◉ colors = depth in cell	Principle: optical system has certain point spread function: if you focus light to objective --> diffraction. Depending on lens --> different spreading owning to diffraction, Result of having small dye and point spread function of system --> you don't see dye in its actual size, but in focal spot size. The inner diameter shrinks as the laser intensity increases	Scheme: ◉405 nm Gaussian beam to restore the bright state, put everything in fluorescence state ◉488 nm dump beam (donut beam), turn everything off except for narrow area in the middle ◉488 nm probe beam, read out what is left in the center of the donut Repeated cycles ➥More intensity in the donut, the sharper the zero point / intensity / dark form in the apex of the donut
◉ Widefield microscope ◉sensitive and flexible ◉Super resolution both in xy and z ◉Sensitivity and flexibility of fluorescence ◉Simple setup and relatively inexpensive				High resolution	less power needed
tion time limited,	◉Time consuming analysis, ◉More and better PhotoActivable and PhotoSwiching FPs needed!, problem!	◉Spontaneous activation of FPs leading to background, ◉Number ◉Labelling density	, ◉Performance of FPs experiment dependend!	Need of stable dyes because of the very high laser power used	switching process is in ms time range, tells you that ◉ every pixel needs to be eliminated for several ms ◉ acquiring an image takes minutes
Even simpler schemes possible?					
membrane rafts/micordomains (10-200 nm)	Following how a **dart** is thrown and hitting target → only measure by stroboscopic method		Actins/filamentous networks		Reversable photoswitchable fluorescence protein dronpa
class 8 superresolution microscopy	class 8 superresolution microscopy	class 8 superresolution microscopy	class 8 superresolution microscopy	class 9 superresolution microscopy	class 9 superresolution microscopy

Donut beam excitation	Structured Illumination Microscopy	Optical Fluctuation	Lifetime measurement	Labe
MINFLUX	**SIM**	**SOFI**	**FLIM**	**Raman Spectroscopy**
Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes	Structured Illumination Microscopy	Super-resolution optical fluctuation imaging	Fluorescence-lifetime imaging microscopy	Raman Spectroscopy
	factor 2 improvement (100nm?)	Limited improvement	same as confocal	same as confocal? (since image with pinhole)
	Static			Static & Dynamic
/	/	depends on # images you make	same as confocal	same as confocal
triangulate position of molecule with very fast electronics that move donut beam along (tracking, static images,...) donut beam determines the steepness intensity change in the donut → determines how accurate you can pinpoint the position of the molecule.	⦿Illuminate the sample under different angles with a certain illumination pattern ⦿ This will give more high frequencies in Fourier space => High freq are for resolution ⦿Reconstructed image has better resolution!	⦿you need fluorophores that have a certain fluctuating behaviour ⦿measure a widefield image in time ⦿from all pixels in time you construct autocorrelationfunctions ⦿The ACF are used to construct the SOFI image => slightly better resolution	⦿excite points in confocal with pulsed laser ⦿you get a intensity-time curve for each pixel => lifetime for each pixel ⦿Lifetime of fluorophore can change depending on surroudings => environment conditions can be imaged with FLIM (viscosity, pH, autofluorescence,...) ⦿Needs to be done at magic angle (L1)	⦿illuminate sample with laser ⦿Most of light is Rayleigh scattered (no energy difference) ⦿Part of incoming photons are Raman scattered (energy difference depending on energy between the binding atoms) ⦿The raman spectrum gives info on what type of bonds are in the spot ⦿Image has spectral info on each pixel ⦿Scattered intensity is higher for high energy photons but it can also destroy the molecule. NIR is less intensity but is less absorbing in bio material!
⦿lower photon load than PALM for some experiments		⦿ On classical standard microscopes, you need to change probes in your sample (if you have something that is expressing GFP you need to change it to dronpa) ⦿ robust against low signal to noise ratio (because autocorrelation/cross correlation calculation) --> in FCS: it's one of the few signals that goes brighter the lower amount of molecules being present. ⦿ improved resolution and background rejection (not correlated, filtered out) ⦿ imaging is fully specified by a clear analytical model	⦿ Can be done with slightly changed confocal ⦿ Lifetime gives extra information about what happens in certain pixels ⦿Allows differentiation between fluorophores with same emissionspectrum!!	⦿Chemical info in images ⦿No photobleaching and non-invasive ⦿No fluorescence needed ⦿Biocompatibility ⦿Better resolution than IR
		⦿ need multiple image acquisitions (more if data is of lower quality) ⦿ limited resolution improvement (2x to 3x for FPs) ⦿ only works with fluorphores that show dynamics on the order of image acquisition time.	⦿ you need pulsed laser	⦿ Raman is very weak signal!! ⦿Because of weak signal needs very long acquisition times ⦿Fluorescence can overwhelm
⦿ ATTO 647N molecule ⦿ dna origamis ⦿ tracking in living E. coli bacteria	/	tubulin network of a 3T3 fibroplast	⦿different autofluorescence species in pollen ⦿Autofluorecence differentiation of stomach tissue ⦿Stem cell differentiation ⦿Viscosity measurement ⦿9 fluorophore measurement	⦿Raman microscopy on lymphocytes
class 9 superresolution microscopy	class 9 superresolution microscopy	class 9 superresolution microscopy	class 10 lifetimes, FLIM, FRET	Class 11 Non-linear and label free

l free techniques and Non-linear techniques (two photon belongs also to this group)			Mobilit	
SERS	**CARS**	**SHG**	**FCS**	**RICS**
Surface-enhanced Raman spectroscopy	Coherent anti-Stokes Raman spectroscopy	Second-harmonic imaging microscopy	Fluorescence correlation spectroscopy	Raster Image Correlation Spectroscopy (Fluorescence fluctuation imaging)
same as confocal? (since image with pinhole)	/	/	Same as confocal	Same as confocal
Static & Dynamic	Static & Dynamic	Static & Dynamic	Dynamic	Dynamic
same as confocal	/	/	Same as confocal, interesting fluctuations in µs to low ms	+-1s per frame 1us per pixel!
⦿enhancement of weak Raman signal by adding metal nanoparticles (NP) to the sample. In the region where these NPs touch, the Raman Signal is enhanced million times	⦿ excite with pump laser (L1) to a virtual state ⦿illuminate then with a second laser (L2) that stimulates emmision for stokes. This brings the waves in coherence ⦿The Probe laser (L1) then excites to another higher virtual state from which emission gives a photon with anti-Stokes signal ⦿The lasers are pulsed to get good Raman signal without too much damage ⦿Allows to image certain bond (mainly lipids)	⦿ Use 2 low energy photons to excite to a virtual state => 2 photons are used to scatter into 1! ⦿You use very short, very intense pulses = high 2 photon probability but no continuous high intensity ⦿Can only be used in noncentrosymmetric systems = mainly highly organised bio nano structures	⦿Confocal used to detect fluo in 1 point ⦿Intensity fluctuations due to fluorophores passing the focus spot => autocorrelation ⦿Allows measuring of diffusion cte D and concentration C!!! ⦿Dual colour FCS possible! (called FCCS)	⦿CLSM image from which a 2D correlation function is calculated ⦿Amplitude = info on # particles ⦿Shape of 2D-CF = info on diffusion ⦿Can be used for mobility mapping ⦿dual colour RICS possible (RCCS)
⦿Stronger Raman signal	⦿No labelling recquired ⦿You can image different bonds to see different types of particles	⦿Label free, Non-invasive, penetration depth (NIR), 3D sectioning ⦿Compatible with other techniques	⦿Confocal microscope ⦿Concentration, stoichometry and mobility! ⦿ Dual color FRAP does not imply interaction => Dual color FCS does!	⦿Different types of info (concentration, diffusion,...) ⦿Mobility mapping = shows how 1 molecule moves in different parts of the cell ⦿ Advantages over point correlation methods: -MUCH less artefacts due to photobleaching -you can do it on any LSM (laser scanning microscopy)
⦿You need to add metal NP => not that liked by bio samples ⦿Slightly different types of spectra ⦿NPS don't spread homogenous in cells	⦿Not background free ⦿No energy absorbed	⦿Only in noncentrosymmetric samples!	⦿ Image one point = not an image at once	⦿Works not that good for very fast fluctuations
⦿eGFP spectrum ⦿TERS	/	/	⦿protein dynamics in nuclei ⦿Binding studies in enzymes ⦿binding affinity (FCCS)	⦿Mobility mapping ⦿HIV assembly
Class 11 Non-linear and label free	Class 11 Non-linear and label free	Class 11 Non-linear and label free	Class 6 Measuring Mobility	Class 6 Measuring Mobility

y measurement techniques

SPT	fluctuation spectroscopy	FRAP
Single-particle tracking	average between FRAP and SPT (FCS, FCCS, RICS belong to this category), also called image correlation spectroscopy	Fluorescence recovery after photobleaching
few nm - 20,30,40 nm	30 ms per frame	Not important
Dynamic	Dynamic	Dynamic
	µs to low ms	Not important
◉Measure in widefield with very good camera (EMCCD) ◉Superresolution by fitting analysis => pinpoint localisation of fluorophores ◉Image particle tracks => get diffusion D for individual particles ◉The speed of diffusion is encoded in spatial correlation function.	◉ method to make signal out of noise -> noise is due to molecules entering and exiting your focus. ◉wide field or confocal	◉ Ensemble Method ◉photobleach a small region of fluosample => fluoresence recovers in that region (get D from that recovery) ◉ Do NOT use photostable fluorophores! ◉Variations: FLIP,PA, CP
◉2D widefield ◉Good for slow diffusion and bright dye in very low concentrations	◉no disadvantages from FRAP ◉detailed information	◉ Widefield ◉easy technique
◉Not 3D diffusion analysis! ◉Fluorescent Protein is mainly used, not that bright ◉Diffusion speed not know beforehand	◉ no single molecule information	◉ Ensemble Mehtod ◉ No concentration info ◉ Local info => no mapping! ◉ Diffusion D needs to be constant in time ◉Dual color frap does not mean interaction! ◉ not realtime ◉ no stoichiometry info ◉ slow diffusion only ◉ need bad fluorophores
◉ Intracellular trafficking of gene complexes through a membrane = compare diffusion velocities for different pathways ◉ studying membrane diffusion ◉ intracellular transfection pathways of viruses ◉Intracellular transport of non-viral gene complexes ◉Transport of gene complexes in extracellular matrices ◉Single-enzyme tracking		◉ HIV-1 integrase enzyme + ledgf (see if HIV binds with chromatine) ◉ xylanase (enzyme that binds to sugars) ◉ endolysin peptidoglycan (can bind to membrane)
class 5 single molecules & Class 6 Measuring Mobility	Class 6 Measuring Mobility	Class 6 Measuring Mobility