OPTICS: MULTIPHOTON MICROSCOPY

presented by

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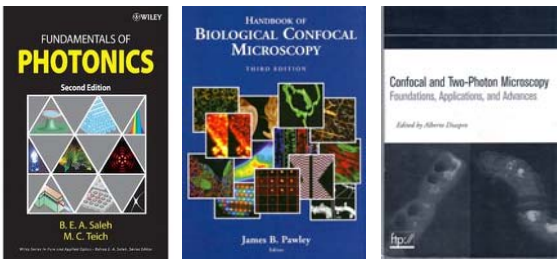
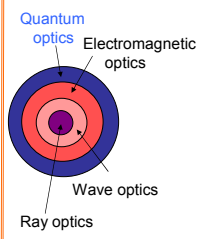
LECTURE OUTLINE

- Background: atoms and molecules
- Photon interaction with atoms
- Two-photon fluorescence microscopy
- Second-harmonic generation microscopy

SOURCE MATERIAL



OPTICS: THEORIES



Ray optics-Limit of wave optics when wavelength is infinitesimally small

Wave optics-provides a description of optical phenomena using scalar wave theory

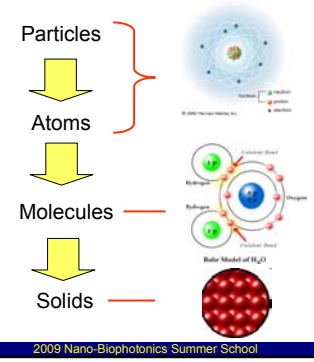
Electromagnetic optics- provides most complete treatment of light within classical optics

Quantum optics-provides a quantum mechanical description of the electromagnetic theory

BACKGROUND

- Light interaction with matter is mediated through electric charges
- Time-varying *E*-field exerts forces on atoms, molecules, and solids
- Interaction is governed by rules of quantum mechanics
- Vibrating electric charges can absorb and emit light

BACKGROUND



SCHRODINGER EQUATION

- Describes behavior of a particle of mass *m*

$$\frac{-\hbar^2}{2m}\nabla^2\Psi(\mathbf{r},t)+V(\mathbf{r},t)\Psi(\mathbf{r},t)=-j\hbar\frac{\partial\Psi(\mathbf{r},t)}{\partial t}$$

Complex wavefunction — potential

- Environment (including interaction with light) is characterized by the potential
- Used to determine allowed energy levels *E*
- More complex systems (e.g., multiple particles) require more complex potential

TIME-INDEPENDENT SCHRODINGER EQUATION

- If *V* is time-invariant equation is simplified

$$\frac{-\hbar^2}{2m}\nabla^2\psi(\mathbf{r})+V(\mathbf{r})\psi(\mathbf{r})=E\psi(\mathbf{r})$$

- Energy levels are determined using a separation of variables technique
- *E* will be
  - Discrete → Atoms
  - Continuous → Free particle
  - Bands → Semiconductors
- System exchanges energy with environment (e.g., thermally, optically) via movement between energy levels

ATOMS

- Energy levels determined by potential energies of electrons (depends on proximity to atomic nucleus and other electrons, as well as forces related to OAM and SAM)
- Simple example is Hyrdogen (single nucleus and single electron)
- Also used in lasing medium (e.g., He-Ne, Ar^+, Kr^+)

MOLECULES

- Combination of 2 or more atoms
- Energy levels determined by the potential energies associated with forces that bind the atoms
- 2 major types of binding are ionic (forms permanent dipole moments) and covalent (no permanent dipole moment)
- Stable when sharing of valence electrons by atoms results in reduction of overall energy

MOLECULES

- 3 types of interactions dictate energy levels
  - rotational transitions → microwave and far-IR region
  - vibrational transitions → IR region
  - electronic transitions → visible and UV region
- Used to make a variety of molecular lasers from gases (e.g., $C0_2$, N_2) to dyes in a solvent (e.g., Rhodamine-6G, Polymethine)

INTERACTIONS OF PHOTONS WITH ATOMS

- Atom can emit or absorb a photon by undergoing upward or downward transition between energy levels, respectively
- We will consider what happens when an atom (with energy levels E_1 and E_2) is placed in an optical resonator
- Atom interacts with photons of a specific mode of frequency ν_o where $h\nu_o=E_2-E_1$
- Studied using QED

INTERACTIONS OF PHOTONS WITH ATOMS

- 3 types of interactions

  - Spontaneous emission

  - Absorption

  - Stimulated emission

SPONTANEOUS EMISSION

- Atom is initially in upper energy level (2)
- Atom decays spontaneously to lower energy level (1)
- Transition releases photon of energy $h\nu \sim h\nu_o = E_2 - E_1$
- Photon energy is added to energy of electromagnetic mode
- Independent of the # of photons that may already be in the mode

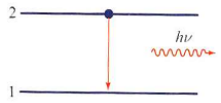


Image Source: Fundamentals of Photonics

ABSORPTION

- Atom is initially in lower energy level (1)
- Radiation mode contains a photon
- Photon is "annihilated" and atom is raised to higher energy level (2)
- Photon induces this process called absorption; depends on photon existing in mode
- For *n* photons, probability for absorption of a single photon is increased by *n* times

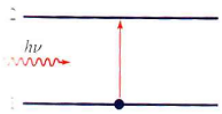


Image Source: Fundamentals of Photonics

STIMULATED EMISSION

- Atom is initially in upper energy level (2)
- Radiation mode contains a photon
- Atom is induced to emit another photon into the same mode
- Emitted photon is a "clone" of the first
- Process is inverse of absorption
- For *n* photons, probability for stimulated emission is increased by *n* times
- Forms foundation for lasers

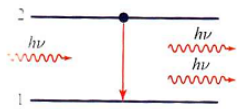


Image Source: Fundamentals of Photonics

LUMINESCENCE

- Atomic or molecular systems can be raised to higher energy levels through excitation from heat, light, electronic impact, sound
- Nonthermal excitation results in a radiation process called luminescence

PHOTOLUMINENSCENCE

- Light emission is induced by absorption of photons
- Glow emitted by some materials after exposure to UV
- Spin-forbidden transition (lifetime is relatively long ~ 1ms to 10s)
- Also occurs with some quantum dots
- Referred to as fluorescence when transition is spin-allowed (lifetime relatively short ~.1 to 20 ns)

Colloidal CdSe quantum dots exposed to UV light

Image Source: Fundamentals of Photonics

FLUORESCENCE MICROSCOPY

- Fluorescence microscopy is commonplace in investigations of living cells and tissues
- Needs input optical energy of the right frequency and (usually) exogenous or endogenous fluorescent probe
- Can be easily carried out using standard microscope architecture
- Provides high contrast and specificity

1PE FLUORESCENCE MICROSCOPY

- Carried out at excitation wavelengths (blue region) that could result in photodamage of biospecimens
- Risk of photobleaching fluorophores in entire volume
- No axial sectioning w/out the use of a confocal pinhole
- Limited penetration depth (~50 μm) into tissues due to absorption from water, melanin, hemoglobin

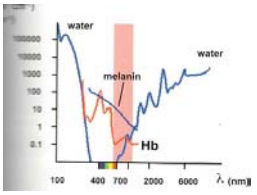
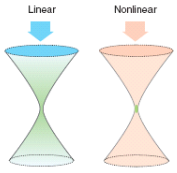


Image Source: Handbook of Biological Confocal Microscopy

NONLINEAR MICROSCOPY

- Nonlinear methods – approaches whereby output intensity is proportional to I^n, where I is the input intensity and n is the number of photons involved in the interaction
- Permits "optical histology"
- Deeper penetration depths (~600 μm compared to 50 μm)
- Reduced photodamage
- Reduced photobleaching



Denk et al, *Nature Methods* **2**, 932 - 940 (2005)

NONLINEAR MICROSCOPY

- 2ph-, 3ph-microscopy
- 2nd-, 3rd-harmonic generation microscopy
- CARS microscopy

COMPARISON OF SETUPS

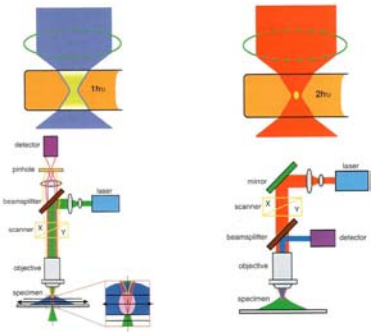


Image Source: Handbook of Biological Confocal Microscopy

HISTORY

- Predicted in 1931 in Maria Goeppert-Mayer's dissertation
- In a typical sunny day, a good 1-ph or 2-ph absorber (molecule)
  - absorbs 1-ph ~ 1/sec
  - simultaneously absorbs 2-ph ~ $1/10^7$ years
- Requires high-photon flux density

HISTORY

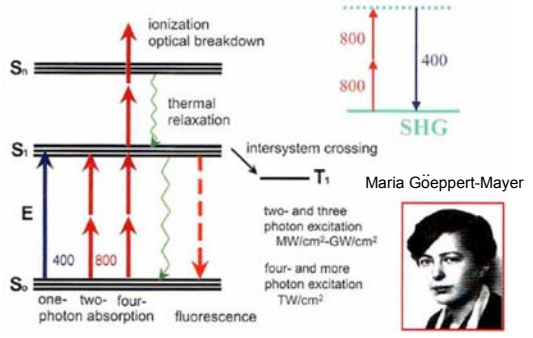
- First experimental demonstration in 1961 was of second-harmonic generation in quartz crystal
- Followed by 2-photon excitation in $CaF^2:Eu^{2+}$ (also in 1961)
- Development of mode-locked lasers made it practical for microscopy tool as demonstrated by Denk *et al.* in 1990

BASIC CONCEPT

- Incoherent nonlinear microscopy techniques: 2-, 3-, *n*-photon microscopy
  - Based on molecular absorption
  - Emission is isotropic and unpolarized
  - Phase information is lost
- Coherent nonlinear microscopy techniques: second-harmonic generation (SHG), third-harmonic generation (THG), coherent anti-Stokes Raman scattering (CARS) microscopy
  - Based on photon scattering
  - Emission is directional and polarized
  - Phase information is maintained

Two-photon excitation/absorption

BASIC CONCEPT



Maria Göeppert-Mayer

Image Source: Handbook of Biological Confocal Microscopy

PROBABILITY FOR MPA

• Probability that a fluorophore at the center of focus of a lens will simultaneously absorb 2 photons (paraxial approx)

$$p_a \propto \sigma_{2PE} \langle P \rangle^2 F_p^{-2} \tau_p^{-1} \left(\frac{NA^2}{2\hbar c \lambda_{ex}} \right)^2$$

2-ph cross-section

Average power

Repetition rate

Pulsewidth

• For optimal fluorescence generation and avoiding saturation, p_a<1, F_p = 100 MHz,<P> = 1-10mW, assuming τ_p=100 fs, and NA =1.4

SPATIAL CONFINEMENT

- For tightly-focused beam, p_a outside focal region falls off with z^{-2n}, where z is the distance from the focal plane
- Results in spatial confinement of fluorescence
- Permits axial sectioning

ABSORPTION CROSS-SECTION

- Units of Goppert-Mayer [1 GM=10^{-58} m^4s/photon]
- Difficult to predict from 1PE cross-section
- Measured experimentally (still challenging)
- Rule of thumb is that 2PE cross-section is peaked at 2x 1PE λ

SELECTION RULES

- Selection rules differ for 2PE compared to 1PE
- Selection rules for 1PE for isolated atoms are forbidden for 2PE
- Usually not true for complex dye molecules due to reduced symmetry and molecular vibrations

PULSE WIDTH

- Reducing pulse width comes with a tradeoff
- Really short pulses (< 100 fs) are sensitive to spreading due to group velocity dispersion
- Usually ~ 100 fs works very well

SOME COMMON FLUORESCENT MOLECULES

TABLE 1 Properties of some common fluorescent molecules[a]

Fluorescent molecule	One-photon absorption peak λ (nm)	One-photon emission peak (nm)	Two-photon excitation peak (nm)	Two-photon action cross-section (GM)	Two-photon absorption cross-section (GM)
Rhodamine B	520	572	840		210
Fluorescein	498	518	780		38
Indo-1 free	345	475		4.5 at 700 nm	12
Indo-1 Ca	335	400		1.2 at 700 nm	2.1
Fura-2 free	362	512		11 at 700 nm	
Fura-2 Ca	335	505		12 at 700 nm	
Bis_MSB	340	420		6.0 at 691 nm	6.3
DiI C-18	500	570		95 at 700 nm	
Coumarin 307	395	488	776	19	
Cascade blue	376, 399	423	750	2.1	
Lucifer yellow	430	533	860	0.95	
BODIPY	480	520	920	17	
NADH	340	430		0.01 at 700 nm	
Dansylhydrazine	336	531		0.72 at 700 nm	
DAPI	345	455		0.16 at 700 nm	

[a] So et al. (2000) and Chapter 4.

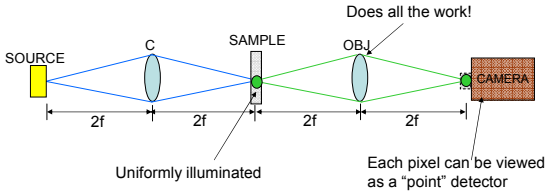
Image Source: Confocal and Two-Photon Microscopy

INTENSITY POINT SPREAD FUNCTION

- Useful to look at intensity PSF, $|h(\rho,z)|^2$
- We can break this down into $|h_{ill}(\rho,z)|^2$ and $|h_{det}(\rho,z)|^2$ for illumination and detection, respectively
- In general, $|h_{ill}(\rho,z)|^2$ and $|h_{det}(\rho,z)|^2$ are not the same b/c of optics
- Also, for fluorescence microscopy λ_{ill} and λ_{det} are not the same

INTENSITY POINT SPREAD FUNCTION

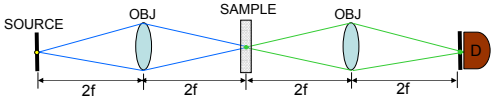
- For conventional fluorescence microscopy, sample is uniformly illuminated by light source and resolution is primarily done by objective



- Image formation is primarily dictated by detection intensity PSF $|h_{det}(x,y,z)|^2$

INTENSITY POINT SPREAD FUNCTION

- Confocal fluorescence microscopy, illumination is by "point" source and detection is by "point" detector
- Resolution depends on both lenses, and hence $|h_{ill}(x,y,z)|^2$ and $|h_{det}(x,y,z)|^2$

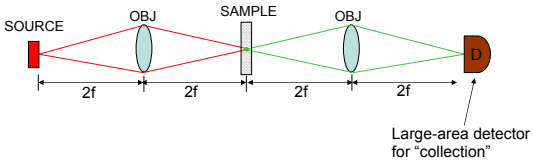


- Requires scanning

$$\left|h_{confocal}(x,y,z)\right|^2 = \left|h_{ill}(x,y,z)\right|^2 \left|h_{\det}(x,y,z)\right|^2$$

INTENSITY POINT SPREAD FUNCTION

- For two-photon fluorescence microscopy there are 2 independent excitation events; p_a is proportional to square of excitation intensity

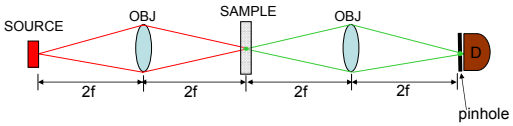


- Excitation alone defines resolution

$$\left|h_{2PE}(x,y,z)\right|^2 = \left|h_{ill\,\lambda 1}(x,y,z)\right|^2 \left|h_{ill\,\lambda 2}(x,y,z)\right|^2 \rightarrow \left[\left|h_{ill}(x,y,z)\right|^2\right]^2$$

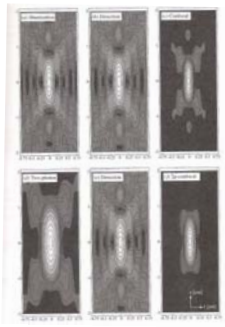
INTENSITY POINT SPREAD FUNCTION

- For two-photon confocal fluorescence microscopy



$$\left|h_{2PE-confocal}(x,y,z)\right|^2 = \left[\left|h_{ill}(x,y,z)\right|^2\right]^2 \left|h_{\det}(x,y,z)\right|^2$$

PUTTING IT ALL TOGETHER



$$\lambda_{1PE-ill} = 488nm$$

$$\lambda_{\det} = 520nm$$

$$\lambda_{2PE-ill} = 900nm$$

Image Source: Confocal and Two-Photon Microscopy

SPATIAL RESOLUTION

- Spatial resolution involves measure of PSF (e.g., Rayleigh criterion)

conventional	$d_{lateral} = \frac{0.61}{NA}\lambda$	$d_{axial} = \frac{2n}{(NA)^2}\lambda$
confocal	$d_{lateral} = \frac{0.4}{NA}\lambda$	$d_{axial} = \frac{1.4n}{(NA)^2}\lambda$
2PE	$d_{lateral} = \frac{0.7}{NA}\lambda$	$d_{axial} = \frac{2.3n}{(NA)^2}\lambda$

λ corresponds to wavelength of detected light

SPATIAL RESOLUTION

- Assuming, $\lambda_{det} = 520nm$

	Lateral	Axial
conventional	240 nm	930 nm
confocal	160 nm	650 nm
2PE	280 nm	1070 nm

- Strength of MPFM comes from its axial sectioning capabilities in thick, highly-scattering, specimens

ADVANTAGES

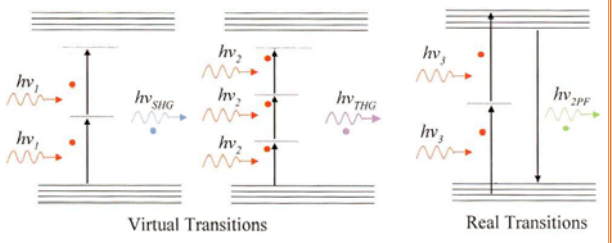
- Strength of MPFM comes from its axial sectioning capabilities in thick, highly-scattering, specimens
- Deeper penetration depths into thick tissues b/c reduced Rayleigh scattering
- Reduced phototoxicity stress on cells permit live cell imaging (same with confocal)

DISADVANTAGES

- $$$ (mainly for fs-lasers)

Second-harmonic generation

BASIC CONCEPT



- SHG, THG → "no energy" is absorbed
- 2PF → energy is absorbed

Image Source: Handbook of Biological Confocal Microscopy

SHG

- Applies to noncentrosymmetric systems, i.e., those that are highly ordered (spatially organized)
- Microtubules, endoplasmic reticulum, bone matrix, collagen fibers

THEORY: SHG

$$P = \chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + ...$$

Induced polarization

$$\begin{bmatrix} P_1(2\omega) \\ P_2(2\omega) \\ P_3(2\omega) \end{bmatrix} = 2 \begin{bmatrix} d_{11} & d_{12} & d_{13} & d_{14} & d_{15} & d_{16} \\ d_{21} & d_{22} & d_{23} & d_{24} & d_{25} & d_{26} \\ d_{31} & d_{32} & d_{33} & d_{34} & d_{35} & d_{36} \end{bmatrix} \begin{bmatrix} E_x(\omega)^2 \\ E_y(\omega)^2 \\ E_z(\omega)^2 \\ 2E_z(\omega)E_y(\omega) \\ 2E_x(\omega)E_z(\omega) \\ 2E_y(\omega)E_x(\omega) \end{bmatrix}$$

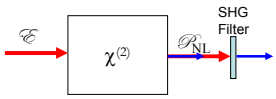
where $\chi^{(2)}=2d_{ij}$

THEORY: SHG

$$P = \chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + ...$$

Induced polarization

P_{NL}



$$\omega_1 + \omega_2 = \omega_3$$

$$\mathbf{k}_1 + \mathbf{k}_2 = \mathbf{k}_3$$

SHG SIGNAL POWER

$$P_{SHG} = \frac{1}{2}\sigma_{SHG} I_{\omega}^2$$

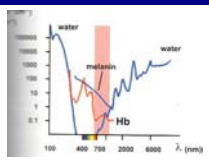
SHG cross-section of an individual dipole

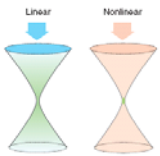
Input power

- Typically, $\sigma_{SHG} << \sigma_{2PE}$
- Results in confinement of SHG signal to sub-femtoliter volume

SHG MICROSCOPY

- Permits “optical histology”
- Deeper penetration depths (~500 μm compared to 50 μm)
- Reduced photodamage
- Reduced photobleaching





Denk et al, *Nature Methods* **2**, 932 - 940 (2005)

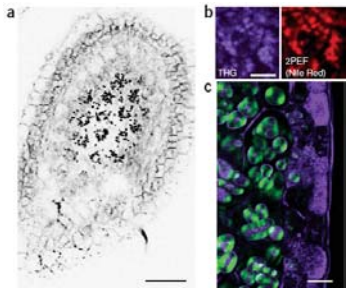
Image Source: Handbook of Biological Confocal Microscopy

COMPARISON TO TWO-PHOTON FLUORESCENCE MICROSCOPY

- Same hardware requirements ($$$)
- SHG signal is coherent while fluorescence is incoherent (polarization and direction)
- Same spatial resolutions (applicable to live imaging)
- SHG is generally label free
- Less heating to specimen since no absorption

MULTIMODE NONLINEAR MICROSCOPY

2PF + SHG + THG



D. Debarre *et al.*, *Nature Methods* **3**, 47 (2006)

NEXT

SHG imaging of collagen-based systems