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Experimental and theoretical investigation of the **Mechanisms of free-electron-mediated modification of biomolecules in nonlinear microscopy**

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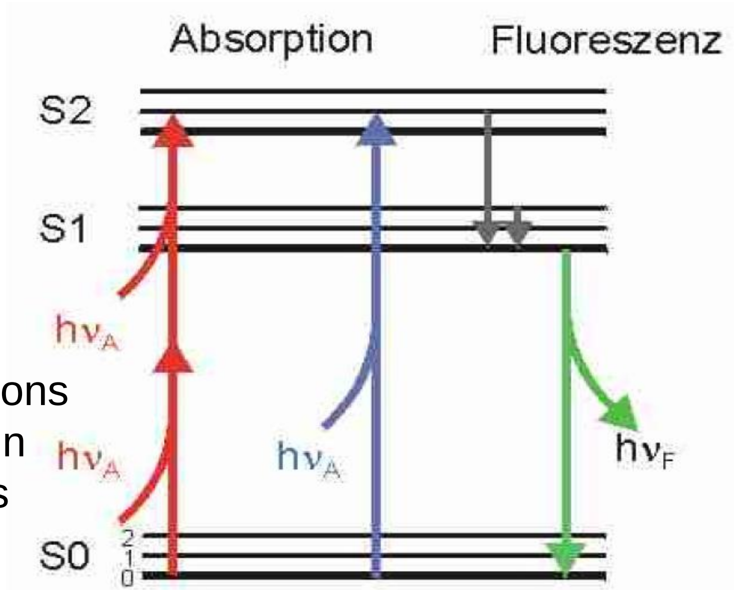
Public Release

Promise of multiphoton microscopy:

Autofluorescence in-vivo imaging inside the body

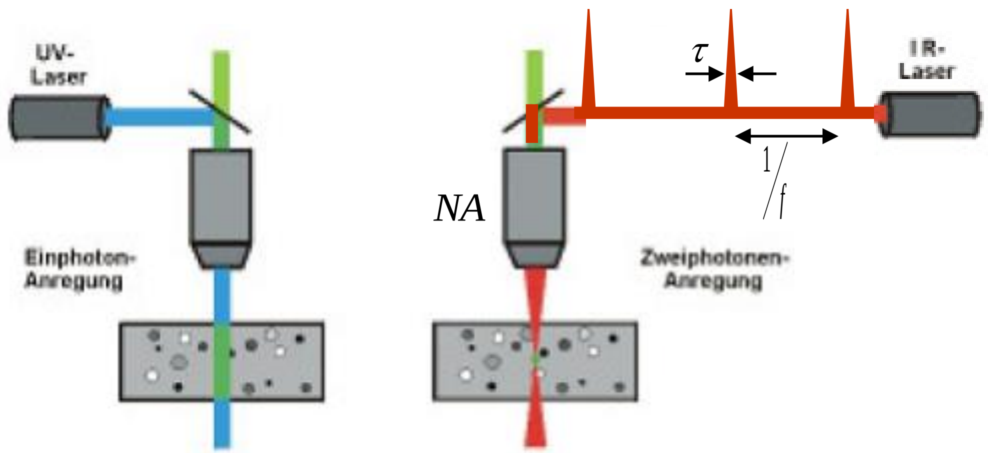
Multiphoton microscopy

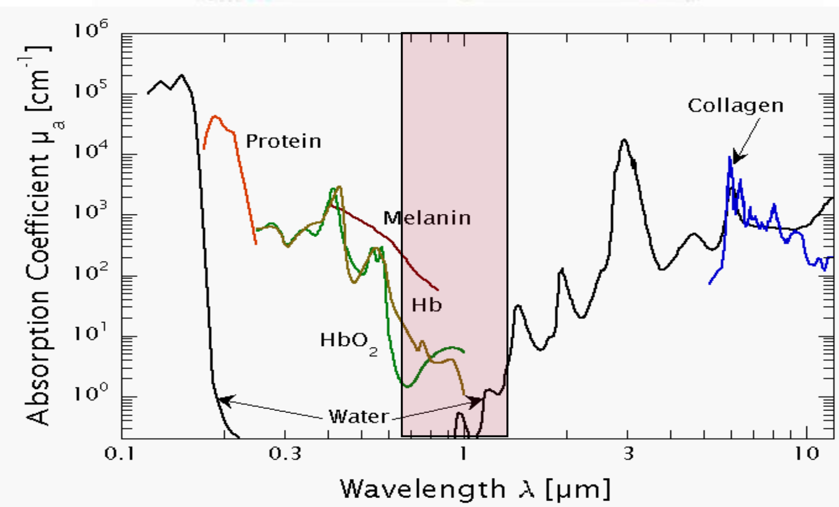
two photons within ≈1 fs

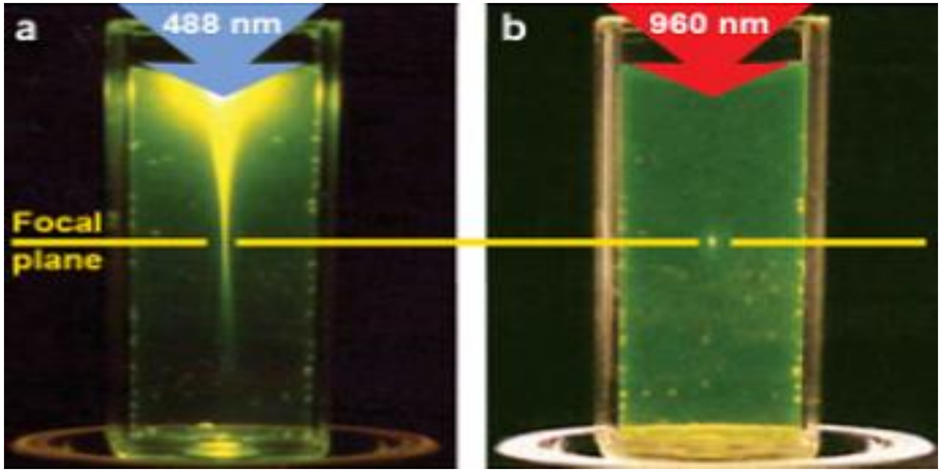


Cw UV laser (confocal microscopy)

Pulse train (80 MHz) from femtosecond oscillator

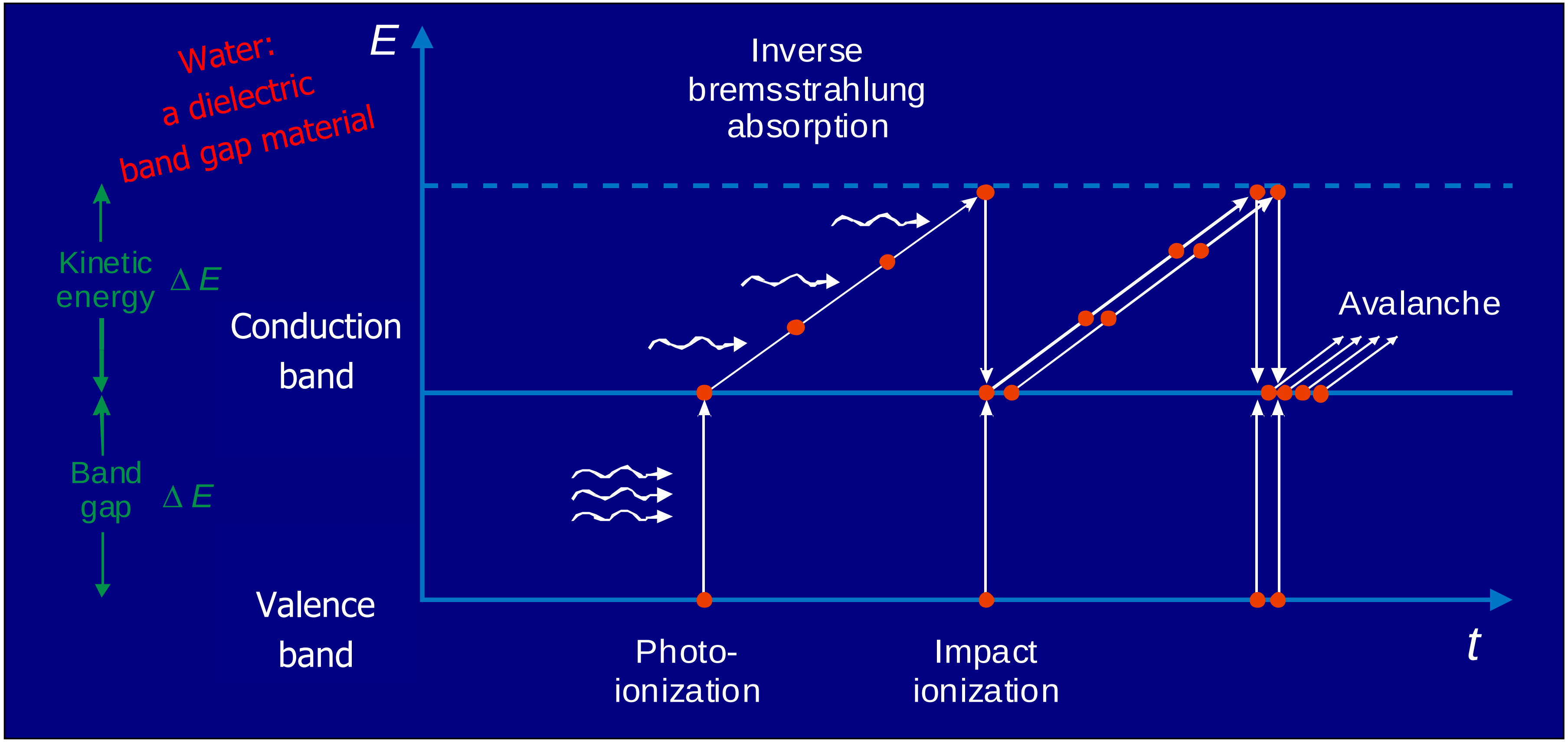






- Up to 0.5 mm penetration depth into scattering tissue
- Well suited for in-vivo investigation of interfaces (skin, lung, intestine)

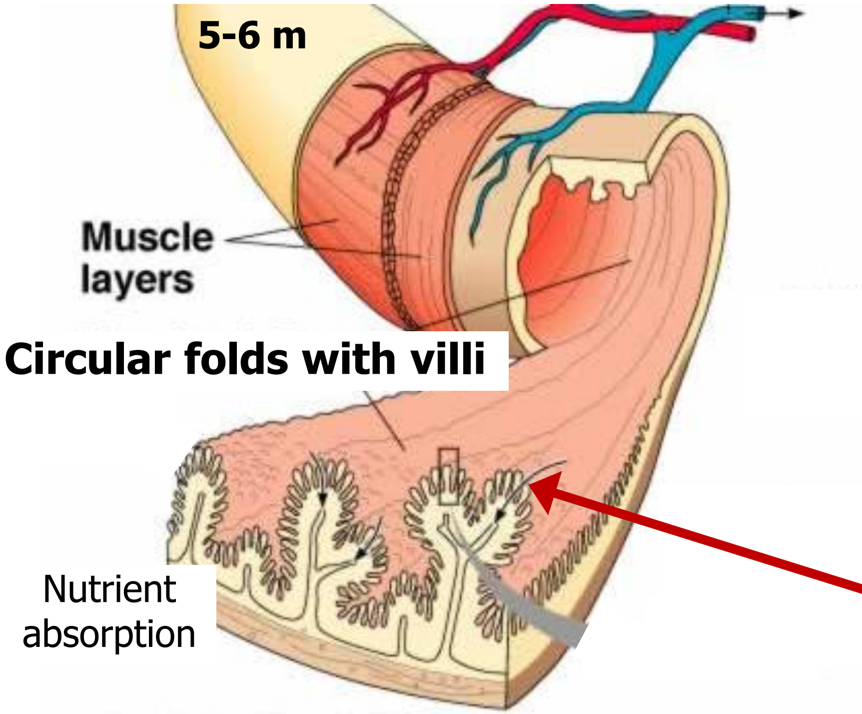
Multiphoton excitation is not far away from plasma formation



- High irradiance is needed for multiphoton excitation
- This implies a certain probability for multiphoton ionization
- That probability increases rapidly $\propto I^k$ (k = order of multiphoton process)

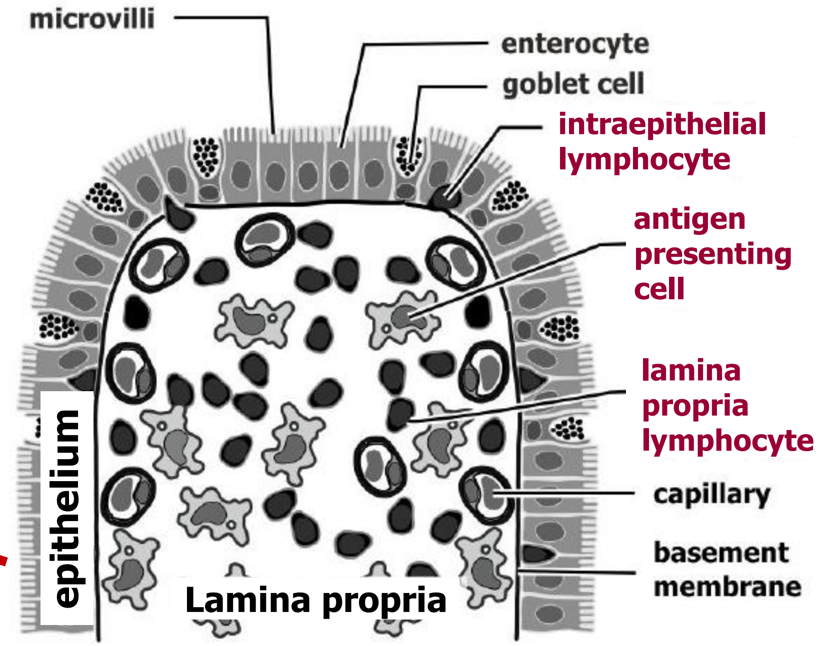
Example: Intestinal mucosa

Small intestine



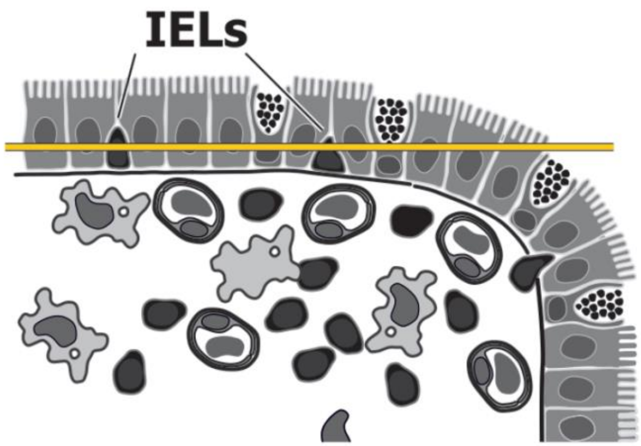
Large surface (400-500 m^2) due to hierarchy of folds

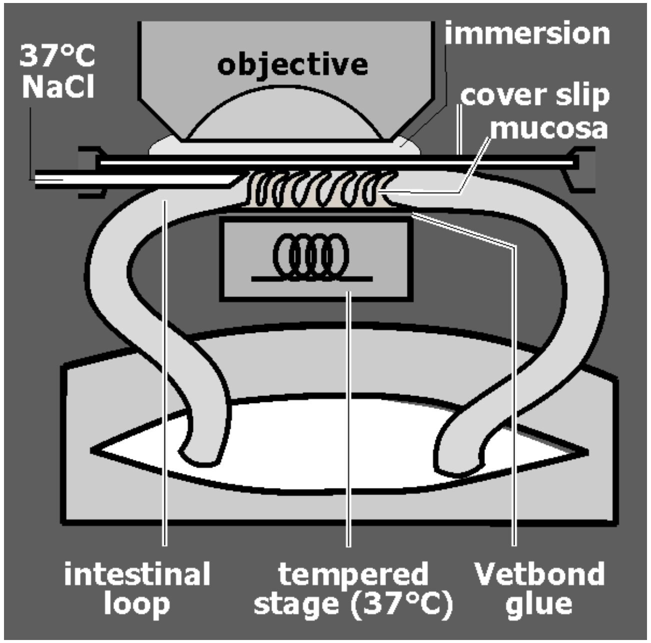
Villus tip



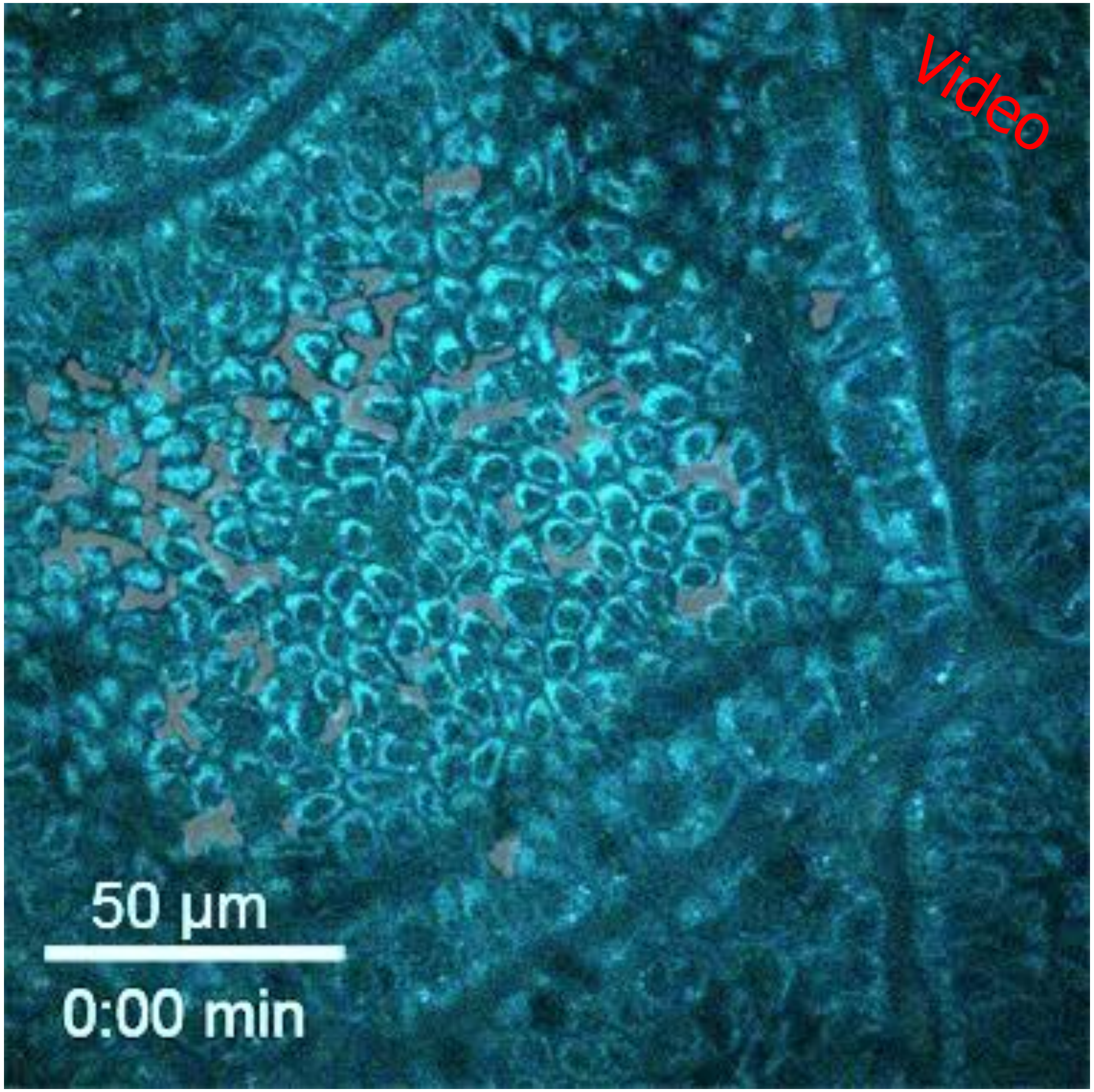
Lymphocytes are parts of the adaptive immune system

Movement of intraepithelial lymphocytes (within 13 min)





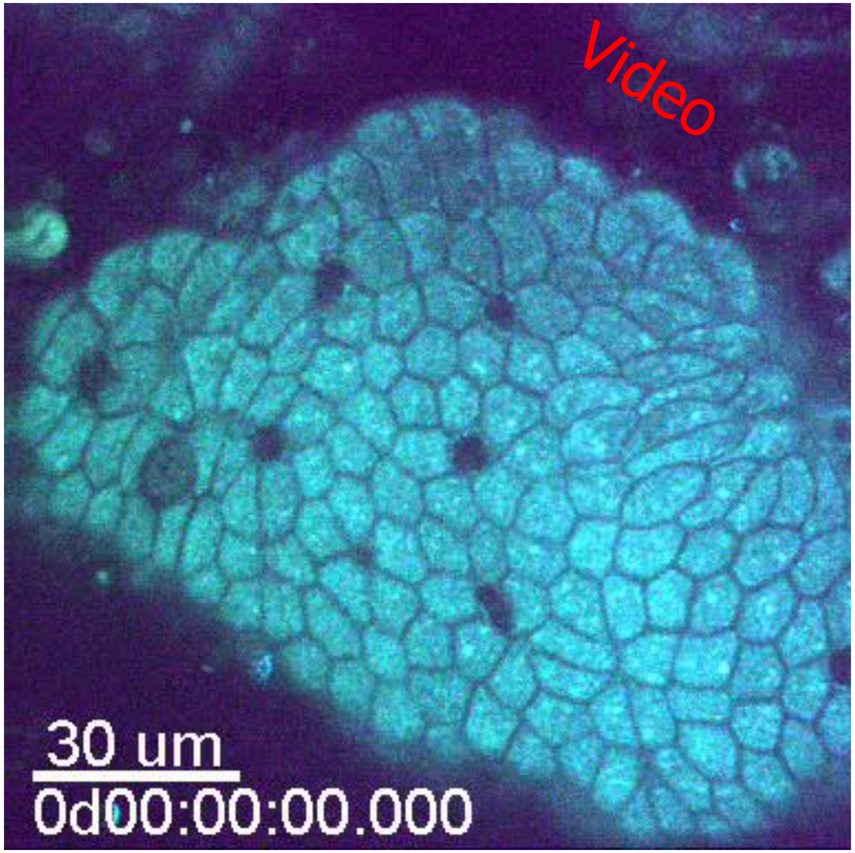
Orzekowsky-Schroeder et al. (2014)
Biomed. Opt. Expr. 10: 3521-3540

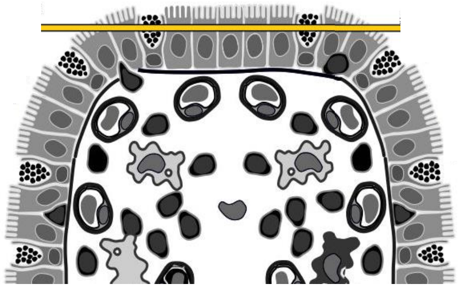


The body‘s immune patrol in action

Twice as much light → photodamage

Repeated (7x) scanning at 38 mW, 730 nm, NA = 1.2 (in total 7500 pulses/pixel)
(2 x the power used for autofluorescence imaging)





Hyperfluorescence sets in at laser powers 1.5-1.6 times larger than used for imaging

Bubble formation follows (dark spots in hyperfluorescent regions)

- Hyperfluorescence increases rapidly once started
- Onset of hyperfluorescence goes along with villus retraction

Orzekowsky-Schroeder et al. (2011) J. Biomed. Opt. 16: 116025, 1-13; König et al. (1996) J. Microsc. 183:197-2014

Outline

Imaging

Femtosecond pulse series

- Evolution of „hyperfluorescence“
  in different tissue types
- Is it hyperfluorescence – or plasma luminescence?
- Quantification of molecular disintegration
  via tracking of gas bubble formation

Manipulation

Single-pulse effects

Nonlinear photochemistry @700-800 nm may introduce fluorescence changes

2-3 photon absorption

Amino acids **NAD(P) H** **Flavins** **Porphyrins**

↓

Hydroxyl radicals H_2O_2 Reactive oxygen species (ROS)

↓

Photoproducts with new fluorescence and nonlinear absorption properties

↓

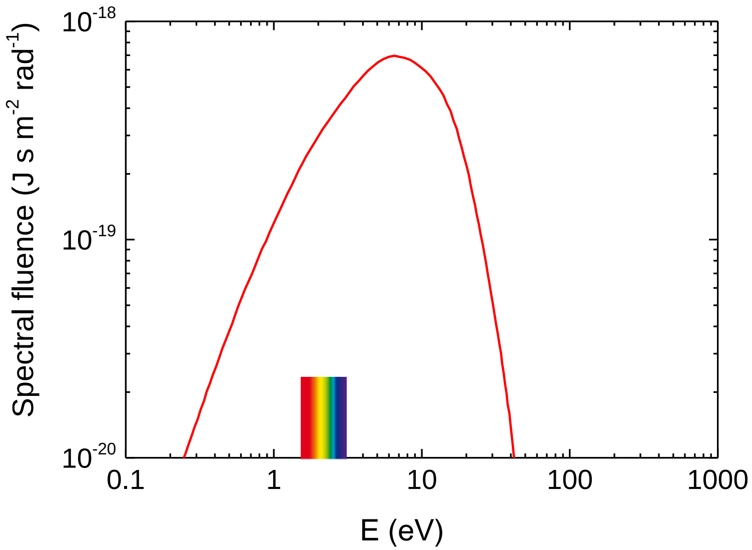
Functional damage, apoptosis, or necrosis

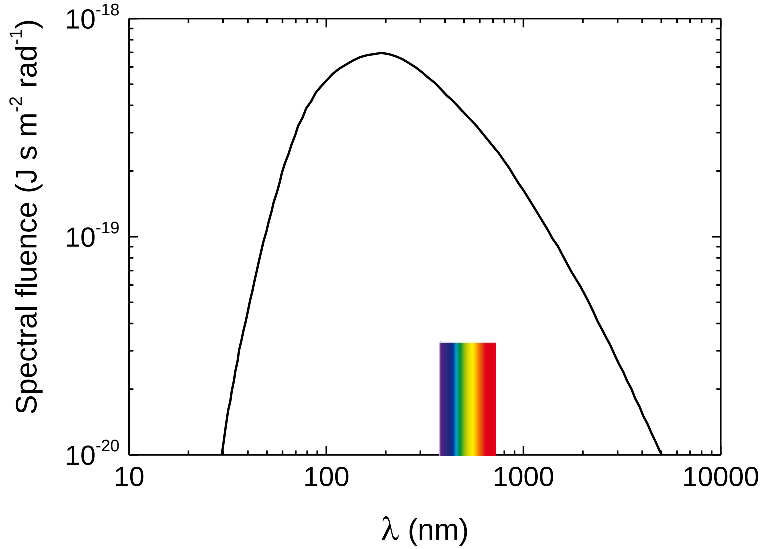
Laser-induced molecular changes may result in changes of fluorescence intensity and life time (detectable via FLIM).

König (2006) in: Handbook of Biological Confocal Microscopy, 3rd ed., Chapter 38, pp. 680-680

Free-electron formation ⇒ plasma luminescence

Bremsstrahlung from the interaction of short laser pulses with dielectrics (SiO_2) near breakdown threshold





Adapted from

Petrov, Palastro & Peñano (2017) Bremsstrahlung from the interaction of short laser pulses with dielectrics, Phys. Rev. E 95, 053209, 1-10

- Conduction band (CB) electrons with energies of a few eV emit visible Bremsstrahlung. It becomes detectable when a burst of 80-MHz fs pulses is applied.
- Recombination radiation would be in the UV range (E_{gap} = 9.5 eV), and in water recombination is, moreover, largely non-radiative.
- Blackbody radiation is emitted from hot, thermalized plasma. However, the *T*-rise during multiphoton imaging is negligible. Bremsstrahlung is emitted before electron energies are thermalized.

Photodamage pathway suggested by the time evolution of fluorescence/luminescence and by FLIM

Nonlinear absorption

Biomolecules

↓ 1

Photochemical changes + Low-density plasma formation

↓ 1

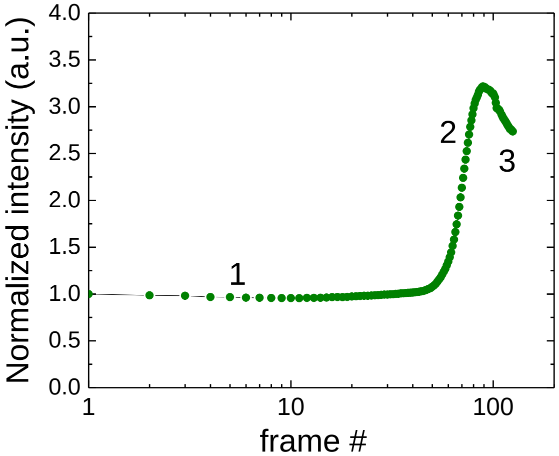
Intermediate photoproducts
with new fluorescence and nonlinear absorption properties

↓ 2

Accelerated photochemistry and plasma formation

↓ 3

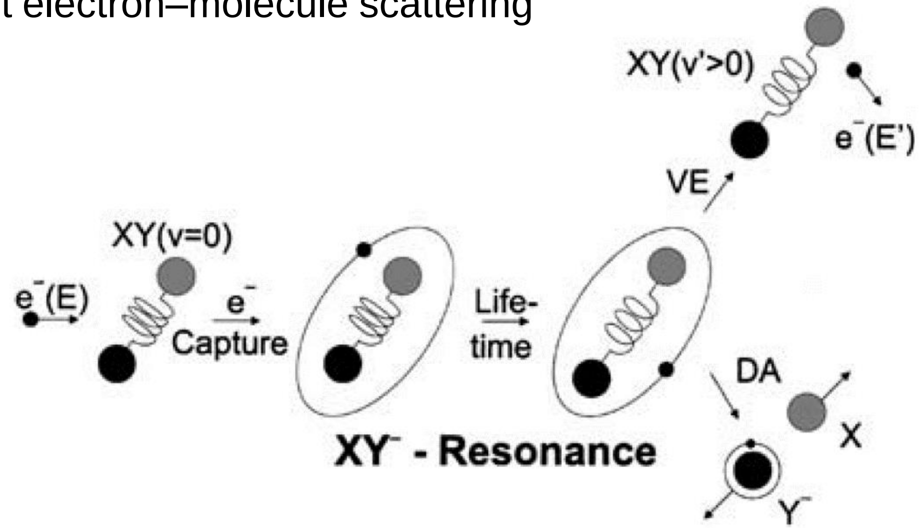
Molecular disintegration and gas bubble formation



Kinetics of gas bubble formation by single spot irradiation with fs laser pulse series

Free-electron-induced molecular disintegration

Dissociative electron attachment
in resonant electron–molecule scattering



Vibrational excitation

➡ Molecular fragmentation and gas bubble formation

Boudaiffa et.al. (2000) Science, 287:1658-1660

Vogel et al. (2005) Appl. Phys. B 81:1015–1047

- Free-electron mediated bond breaking → molecular fragmentation
- Cumulative molecular fragmentation during fs pulse series produces long-lived bubbles containing non-condensable gas
  (different from vapor bubble produced by a single laser pulse)

Vapor bubbles in water ⇔ gas bubbles in tissue

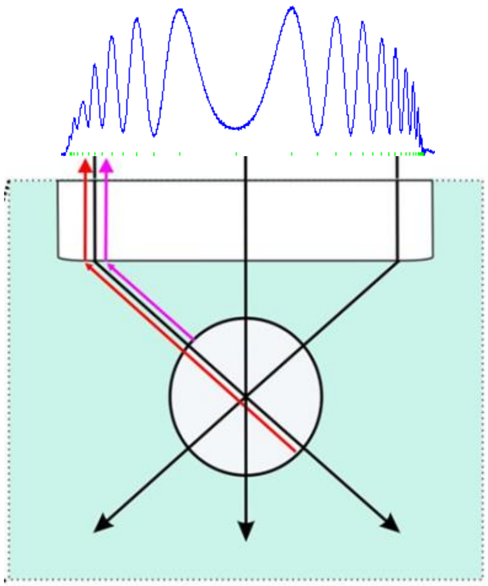
Vapor bubble formation in water

- A bubble is formed, when the focal temperature exceeds the superheat threshold (spinodal limit) ⇒ phase explosion.
- After the phase explosion, the bubble expands beyond equilibrium and initially oscillates.
- Oscillations are damped, and an equilibrium between plasma-mediated vaporization and condensation evolves.
- The bubble disappears immediately at the end of the pulse series

Gas bubble formation in cells & tissue

- Gas bubbles in tissue are formed by *cumulative* molecular disintegration.
- The gas bubble threshold in cells is much lower than the vapor bubble threshold in water. It is closely linked to biomolecular changes.
- The gas bubble grows continuously during the pulse series and vanishes via dissolution of the gas content ⇒ long bubble life time.
- Quantification of gas formation bears info on molecular disintegration rates.

Measuring temporal evolution of bubbles from pulse series by combined interferometry and high-speed photography



- Bubble size evolution *R*(*t*) provides information on the rates at which biomolecules disintegrate into volatile products

⇒ Investigate *R*(*t*) for pulse series at various pulse energies as a function of wavelength

High-speed video
at 100,000 frames/s

- High-speed photography provides a grid of reliable benchmark values for *R*
- Interferometry provides precise info on the radius change d*R*/dt

Radius evolution $$R(t) = R_{Photo} + \int \dot{R}_{Interferometry}\, dt$$

One benchmark photo
every 800 laser pulses
in a 80 MHz train

Evaluation of the mass of altered material in the bubble from the bubble volume

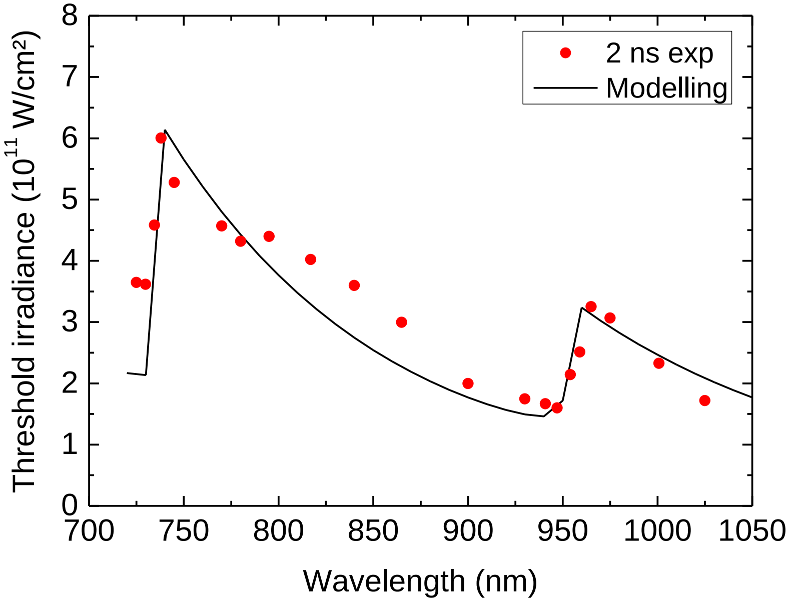
- Equilibrium between internal gas pressure and external pressure is assumed (This simplification is valid for small growth rates).
- Internal pressure = gas pressure from molecular disintegration.
- External pressure = hydrostatic pressure + *p*(surface tension) + *p*(restoring force of cellular matrix).
- Mechanical properties of biological medium must be considered.

- Interferometry and modeling tools for single-shot-produced micro- and nano-bubbles ($R_{max} \to 0$) have already been developed (talk at last review meeting).

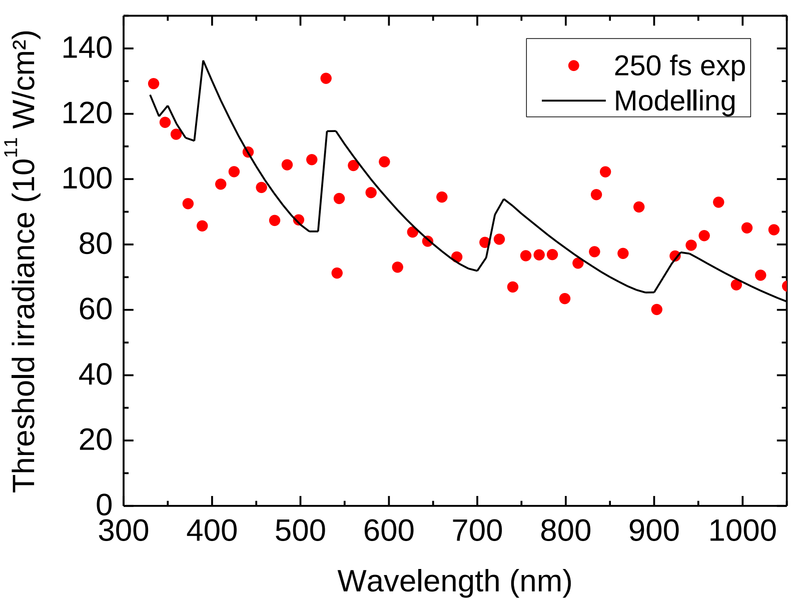
Vapor bubble formation by single-pulse irradiation: $I_{\mathrm{th}}\ (\lambda)$

Optical breakdown threshold spectra for single pulses in water

Nanosecond breakdown



Femtosecond breakdown



- Steps indicate multi-photon initiation
- Separation of the peaks proves the existance of an intermediate energy state E_{ini} at the solvated electron level that can be directly addressed from the valence band

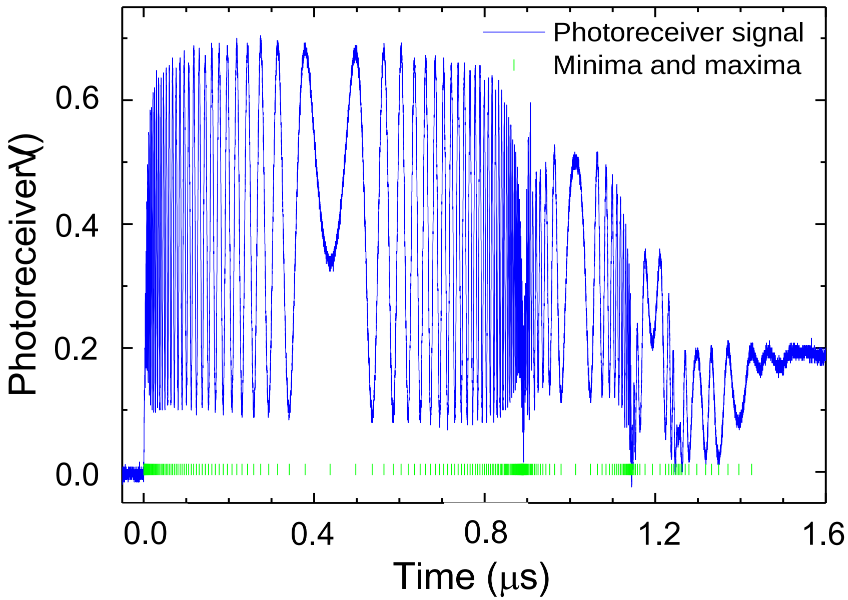
- Decrease of I_{th} with increasing λ indicates dominant role of avalanche ionization
- Breakdown model provides good fit for 1 fs effective Drude collision time

N. Linz et al. (2015) Phys. Rev. B 91:134114, 1-10

N. Linz et al. (2016) Phys. Rev. B 94: 024113

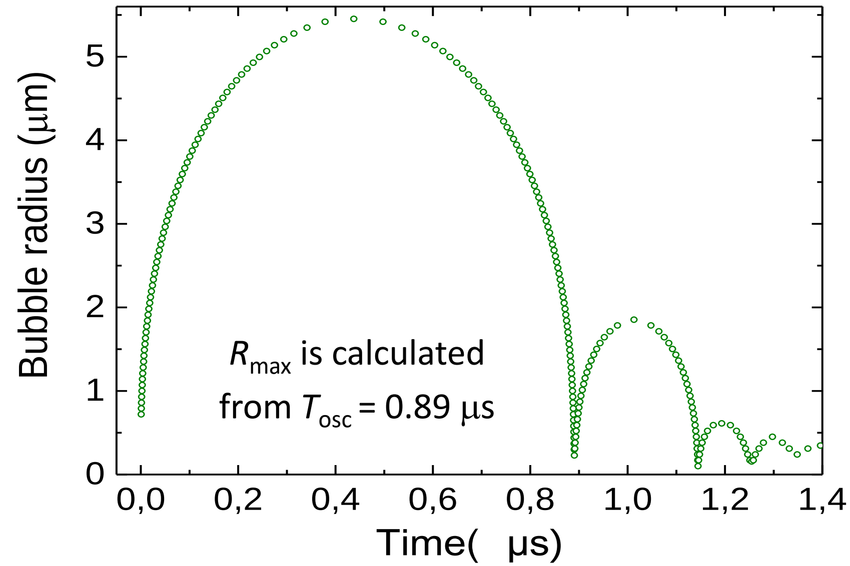
Interferometry and modeling for single-shot produced bubbles

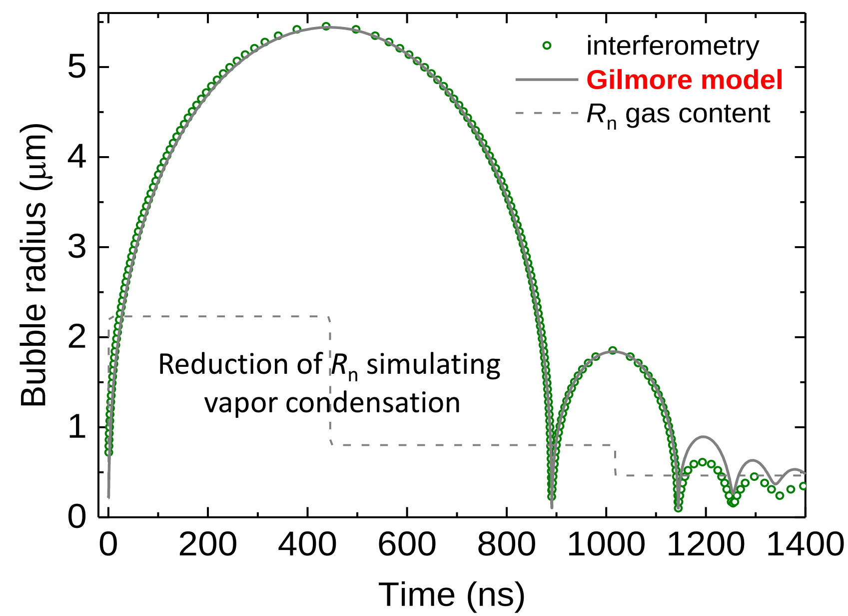
Interferometry signal



R_{max} = 5.4 µm

$R(t)$ curve

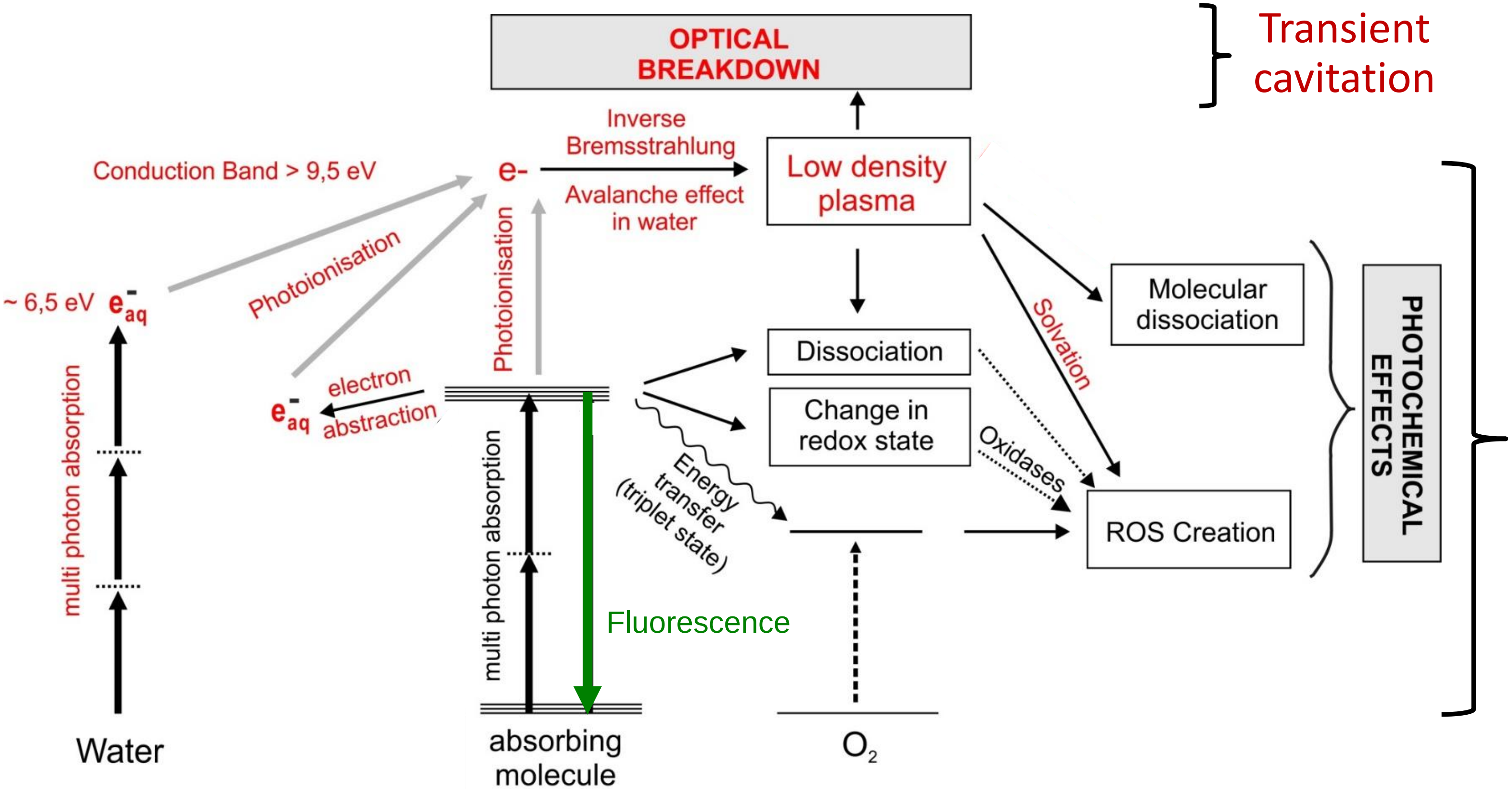




- Spatial resolution: < 5 nm
  Fringe max to min: 70 nm
- Temporal resolution: 160 ps
- Maximum detectable bubble wall velocity: 440 m/s

Conclusions

Pathways of nonlinear photo-modification of biomolecules in aqueous media



Transient cavitation

Gas bubbles

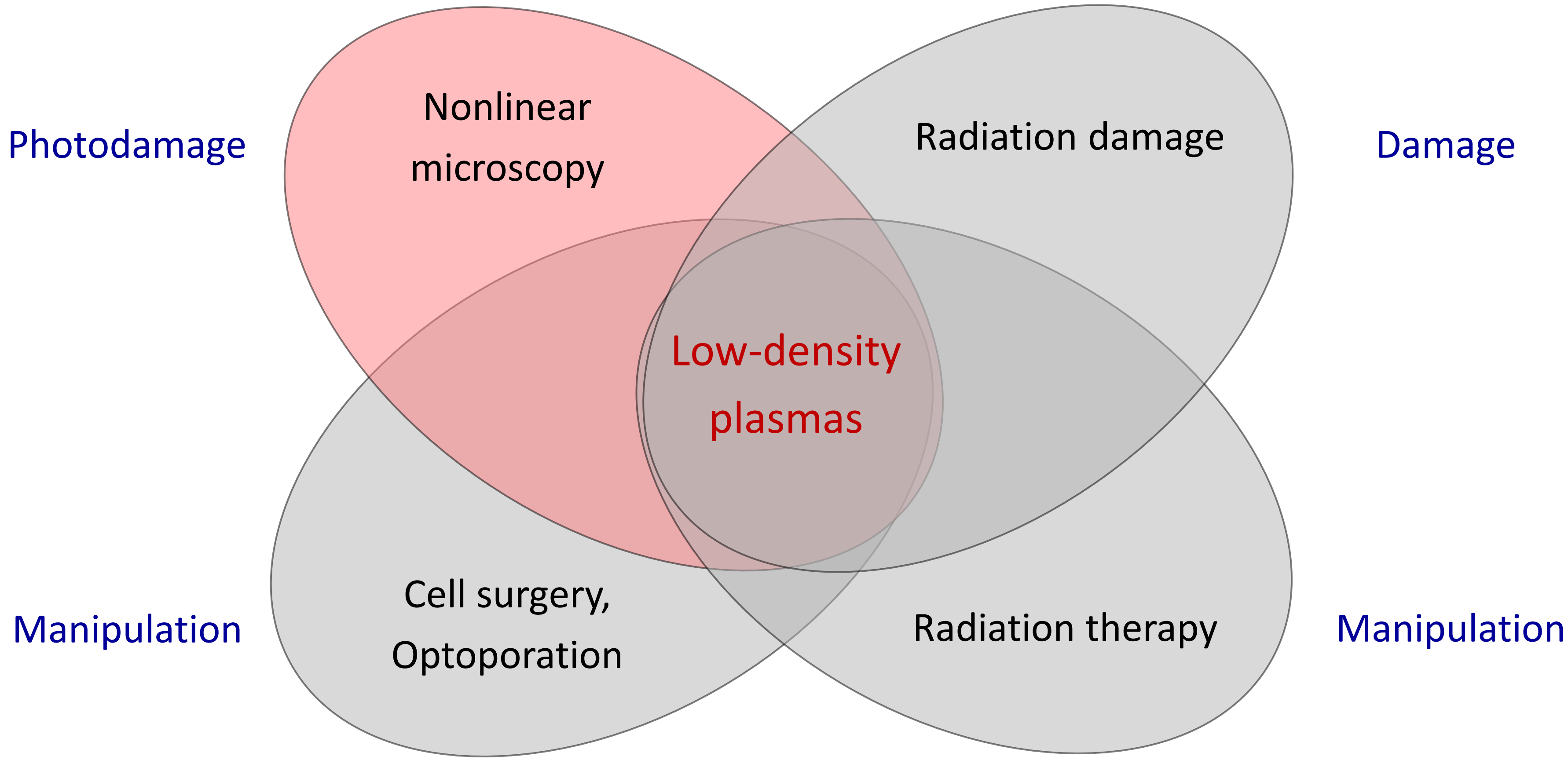
Plasma luminescence

Hypo- & Hyper-fluorescence

Functional damage & Apoptosis

Modified version of a scheme in Debarre et al. PLoS ONE 9, e104250 (2014)

Free-electron-mediated modifications



Free electrons are relevant as part of various **damage** mechanisms and can also be employed for **useful modifications**

Conclusion

- We have collected more pieces of the puzzle describing modifications of biomolecules by nonlinear photochemistry and low-density plasma produced by MHz fs pulse series.

- A coherent picture of molecular interaction mechanisms will support optimum use of the photon budget in nonlinear microscopy, and establish new opportunities for the manipulation of biomolecules.

Thank you from the project team

PI: Alfred Vogel, FOSA, FSPIE

Co-PI: Norbert Linz

Experiments: Sebastian Freidank

Theoretical Modeling: Joe Liang

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