another method developed (doesn't need as much computation)

4. Stimulated Emission Depletion Fluorescence Microscopy (STED)

- uses non-linear depletion of fluorophores from excited state by stimulated emission.

1. focused excitation laser pumps to excited state

2. 2nd "donut" beam triggers stimulated emission of excited molecules = quenches
   but in region around original excitation spot

   This confines fluorescence to small region at center of donut.

3. The donut beam is just 2 laterally offset beams.

   - time delay between excitation and stim-emission.

   ie, fluorescence quenched in outer regions slightly, small amount.
Fig. 1. Energy levels of a typical fluorophore.

Fig. 3. Population probability $n_2(\nu)$ of $L_2$ after Gaussian STED-beam pulses of peak intensities of 3.4, 34, 170, and 1300 MW/cm$^2$ for curves a, b, c, and d, respectively, have left the focal region. (The computational error of the numerical data is less than 0.1%. These curves and curve a of Fig. 4 have been calculated with a density of 150–200 points per curve.)

Fig. 4. PSF’s for the STED fluorescence microscope with $\Delta \nu = 3.9$ (curve a) and the confocal (curve b) and conventional scanning microscopes in the focal plane.
Fig. 2. Principles of a STED fluorescence scanning microscope. An excitation beam and two offset STED beams are focused into the object for excitation and stimulated emission, respectively. The spontaneously emitted light is recorded in a (point) detector. We accomplish imaging by scanning the beams with respect to the object. Two additional STED beams are used for enhancing the lateral resolution in the direction perpendicular to the plane of the scheme. For clarity the lenses for focusing the laser beams into the pinhole plane are not shown.

From Stelzer and W. Wiesmann. Optics Lett. 19(11), 1994. 780-782
STED probe profile

Excitation spot (2D, left), doughnut-shape de-excitation spot (center) and remaining area allowing fluorescence (right).

Intensity profiles. Top: excitation light; center: de-excitation light; bottom: remaining fluorescence.
Stimulated Emission Depletion (STED)

Send to a dark state

\[ FL = \frac{FL_0}{1 + \frac{I_{STED}}{I_s}} \]
STED microscopy

Excitation    STED pattern    Effective PSF
\[ \div \] = ?

Hell 1994, Hell 2000
Fig. 5. Intensity maximum versus the FWHM of the effective PSF of the STED fluorescence microscope.
.

PSF can be significantly reduced.

**ASIDE:**

\[
\text{spatial freq } \nu = \frac{2 \pi f \text{ NA}}{\lambda} \quad \left( d_x = \frac{61 \lambda}{\text{ NA}} \right)
\]

2 STED beams separated by about

\[ \Delta y = 3 \lambda \]

advantage — they can be done in "real" time \(\uparrow\)

good review of super-res. Mics:

Multiphoton microscopy

- At very high photon densities, it becomes possible for two or more photons to be simultaneously absorbed.
- Each multiple absorption induces a molecular excitation of a magnitude equivalent to the sum of the absorbed photon energies.

http://www.aep.cornell.edu/drbio/MPE/mpe.html
Multi-photon fluorescence: Basic principles

- Multi-photon excitation is a nonlinear process
- Because two photons are required for each excitation, the rate of two-photon absorption depends on the square of the instantaneous intensity.
- Because of the large intensities required, high power lasers providing very short pulses (~100 fs) are used, so that peak intensity is high, but average power doesn’t damage the specimen.
- We have photon flux densities sufficiently high for multiple photons to arrive “simultaneously” (in $10^{-15}$ s) at an excitable molecule (of $10^{-16}$ cm$^2$ cross section) only at the focus point of a beam.
- The probability that a given fluorophore at the center of a focused beam absorbs a photon pair during a single pulse is

$$n_a = \delta \langle P \rangle^2 F_p^{-1} \left( \frac{\pi \ast NA^2}{hc\lambda} \right)^2 \xi$$

- $\delta$ is the two-photon absorption cross-section
- $\langle P \rangle$ is the average power
- NA is numerical aperture
- $F_p$ is the repetition frequency

$$\xi = \frac{\langle p^2 \rangle}{\langle p \rangle^2}$$ is known as the two-photon advantage
Figure 2 Localization of excitation by two-photon excitation. (a) Single-photon excitation of fluorescein by focused 488-nm light (0.16 NA). (b) Two-photon excitation using focused (0.16 NA) femtosecond pulses of 960-nm light.
Two photon vs confocal microscopy

Figure 1. Imaging in Scattering Media
Without multiphoton excitation, one has to choose between resolution and efficient light collection when imaging in scattering samples. Nonlinear excitation imaging lifts that constraint as is illustrated here in a comparison to confocal 1-photon imaging (the scan optics are omitted for clarity, but see Figure 2). Typical fates of excitation (blue and red lines) and fluorescence (green lines) photons. In the confocal case (left), the excitation photons have a higher chance of being scattered (1 and 3) because of their shorter wavelength. Of the fluorescence photons generated in the sample, only ballistic (i.e., unscattered) photons (4) reach the photomultiplier detector (PMT) through the pinhole, which is necessary to reject photons originating from off-focus locations (5) but also rejects photons generated at the focus but whose direction and hence seeming place of origin have been changed by a scattering event (6). Excitation, photobleaching, and photodamage occur throughout a large part of the cell (green region). In the multiphoton case (right), a larger fraction of the excitation light reaches the focus (2 and 3), and the photons that are scattered (1) are too dilute to cause 2-photon absorption, which remains confined to the focal volume where the intensity is highest. Ballistic (4) and scattered photons (5) can be detected, as no pinhole is needed to reject fluorescence from off-focus locations.

From Denk and Svoboda, Neuron.
Figure 5  Components of a multiphoton microscope.  (a) Pulse train from a laser mode-locked Ti:S at 80 MHz. (b) Pulses out of the laser typically have a FWHM duration of 100 fs and a spectral FWHM (c) of ~10 nm. (d) Schematic of a MPM system (see text). (e) Rays generated at the focus deep in a specimen may be scattered and enter the objective lens as skew rays, resulting in diverging epifluorescence.  (f) Comparison of PMT photocathode efficiencies.
Figure 6. Resolution along the z-axis for two-photon and three-photon excitation.
Figure 1 | Nonlinear optical microscopy. (a) Jablonski diagram, illustrating two-photon absorption (2PA), second-harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS). Note that in second-harmonic generation and Raman scattering no actual electronic excitation takes place. (b) Spatial confinement of signal generation with nonlinear excitation. Visible ('blue-ish') light is used for excitation in single-photon microscopy, whereas near-infrared ('red-ish') light is used in 2PLSM. In single-photon microscopy an entire cone of fluorescence light (green) is generated, whereas nonlinear signal production is localized to the vicinity of the focal spot. (c) Generic nonlinear laser-scanning microscope. A laser source provides near-infrared ultrashort pulses; intensity and beam size are adjusted before coupling the laser beam to the microscope. The focal lengths of the scan lens ($f_s$), the tube lens ($f_t$) and the objective ($f_o$) are indicated. Two-photon excited fluorescence (2PEF), which is isotropically emitted (inset), can be collected in epi- and/or trans-collection mode, using whole-area detection by photomultiplier tubes (PMTs). Forward-directed optical-harmonic and Raman signals are detected in transcollection mode in transparent samples. For in vivo experiments epicollection is used exclusively.
Microscopy Through the Centuries

\[ d = \text{constant} \cdot \frac{\lambda}{\text{nsin}\Theta} \]

To get better resolution:

1) Reduce \( \lambda \)
   - *electrons, x-rays*

2) Increase \( \text{nsin}\Theta \)
   - *better lenses, “oil”*

3) Decrease constant
   - *confocal*

4) Take away lenses
   - *near field*
   - *scanned tip*
Near Field Scanning Optical Microscopy (NSOM)

Betzig, Isaacson, Lewis and Harootunian, 1986
Power transmission through a metallic screen

Fig 5: Calculation of the power transmitted through an aperture in an infinitely thin, perfectly conducting screen. The incident radiation is assumed to be a polarized plane wave incident normal to the aperture plane. The electric field is parallel to the x-axis, and the magnetic field is parallel to the y-axis. The intensity plot shows the power at a distance of $R/10$ from an aperture of radius $R$ where $R = \lambda/50$, $\lambda = 500$ nm (from ref [16]).

ALUMINUM LETTERS fabricated on a silicon nitride substrate imaged with a near field optical microscope. The full horizontal scale is 550 nm, the wavelength of the light being used.

M. Isaacson and E. Betzig, Cornell University
Effect of Distance on Spatial Resolution

M. Isaacson, et al. 1990
Point Spread Functions for Microscopy

\[ \text{PSF}(r) = |\mathcal{F}[A(\rho)]\mathcal{F}[A(\rho)^*]| \]

Far Field

\[ \text{PSF}(r) = |A(\rho)A(\rho)^*| \]

Near Field

From: M. Isaacson, et.al, Ultramicroscopy (1992)
Near Field Scanning Optical Microscopy (Reflection)

aluminum on silicon