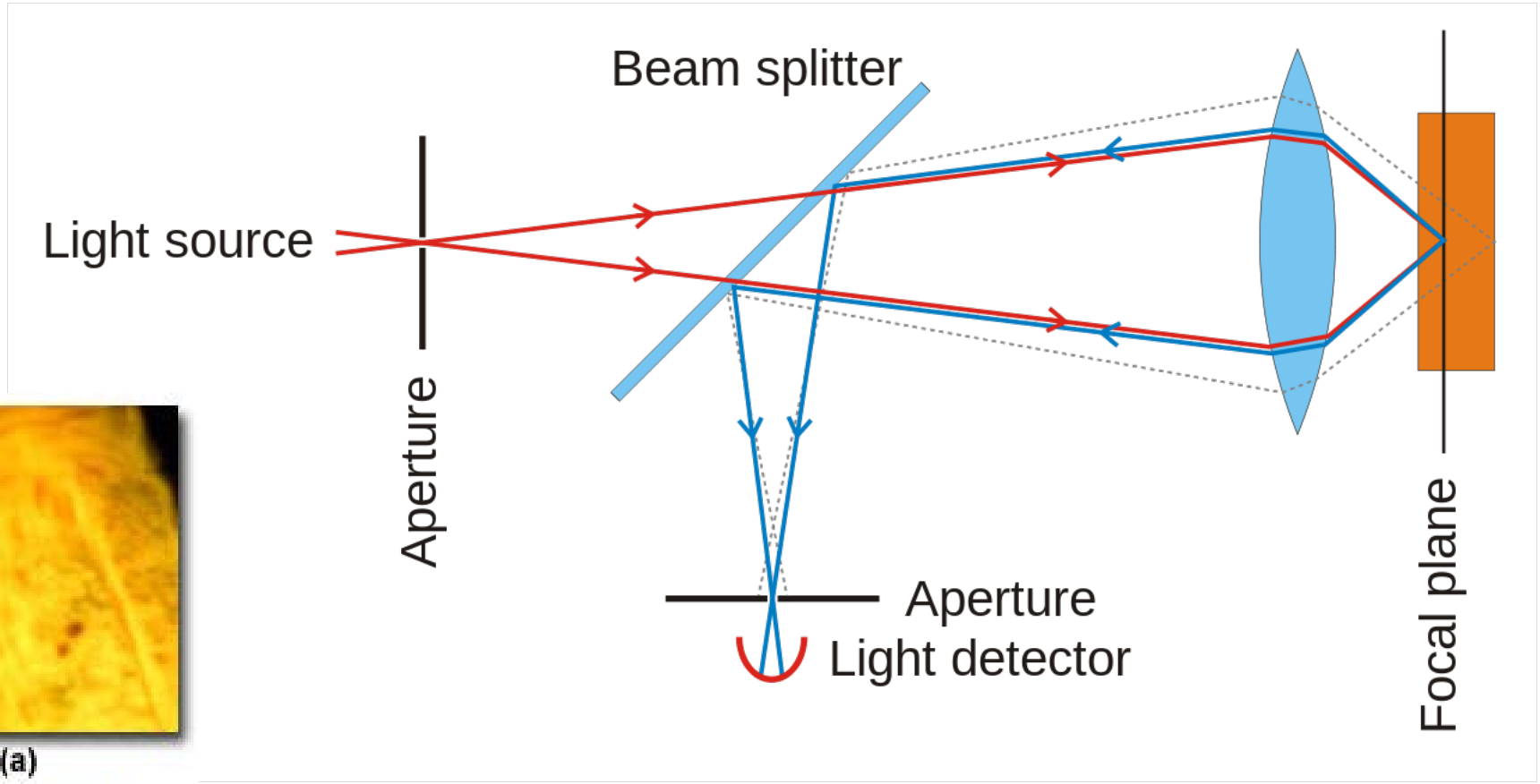
Confocal, hyperspectral, spinning disk

Administrative

- HW 6 due on Fri
- Midterm on Wed
  - Covers everything since previous midterm
  - 8.5 x 11” sheet allowed, 1 side
- Guest lecture by Joe Dragavon on Mon 10/30

- Last class
  - FLIM
  - Confocal
- This class
  - More confocal
  - Hyperspectral imaging
  - Spinning disk confocal



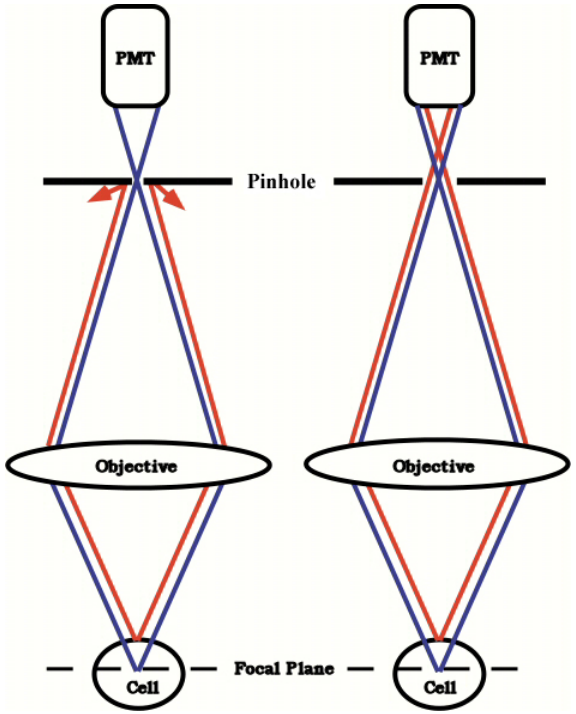
(a)

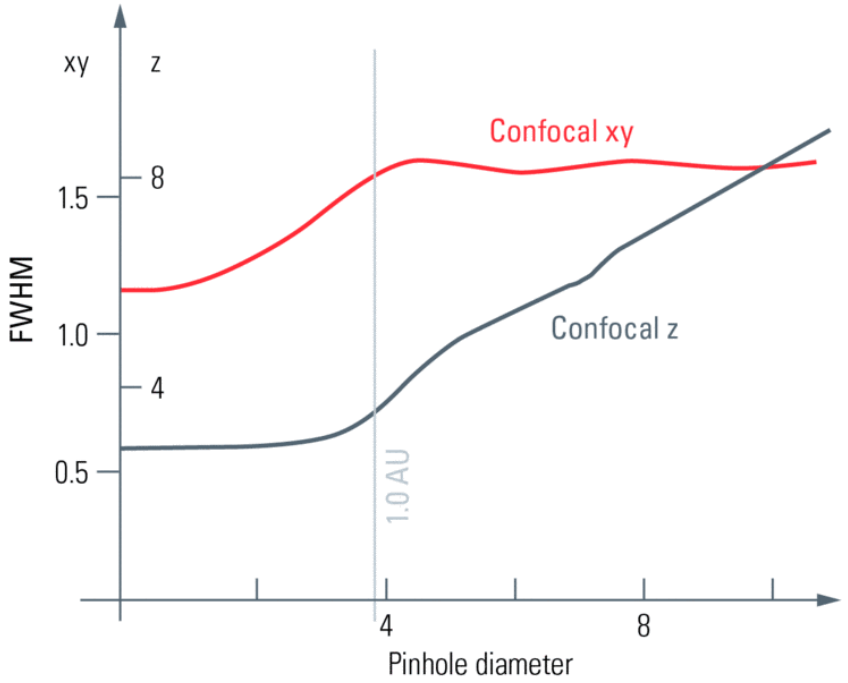
(d)

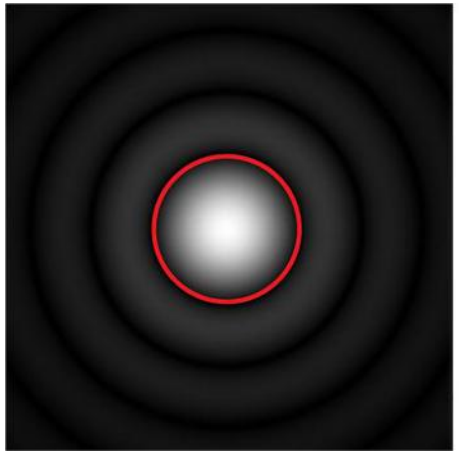
$$d_{x,y} = \frac{0.4\lambda}{NA} \qquad d_z = \frac{1.4\lambda \mathrm{n}}{NA^2}$$

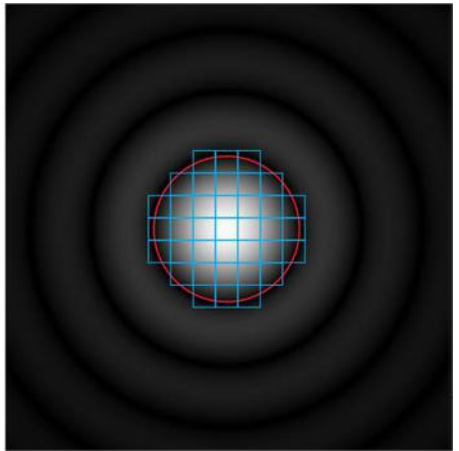
Pinhole size effects

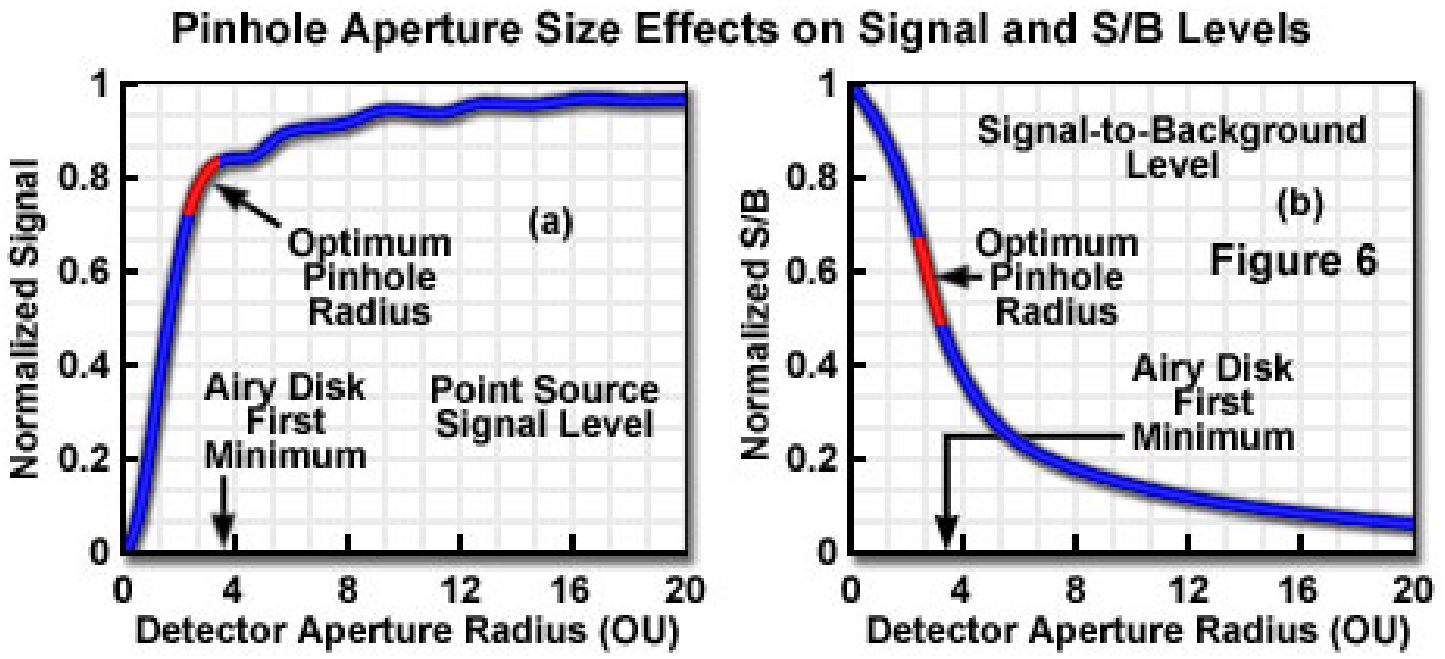
- Decreasing size ->
  - Sharper images
  - Lower light intensity
  - Better z resolution
- Better resolution is not necessarily better. Have to weigh in photostability, sample thickness, etc…





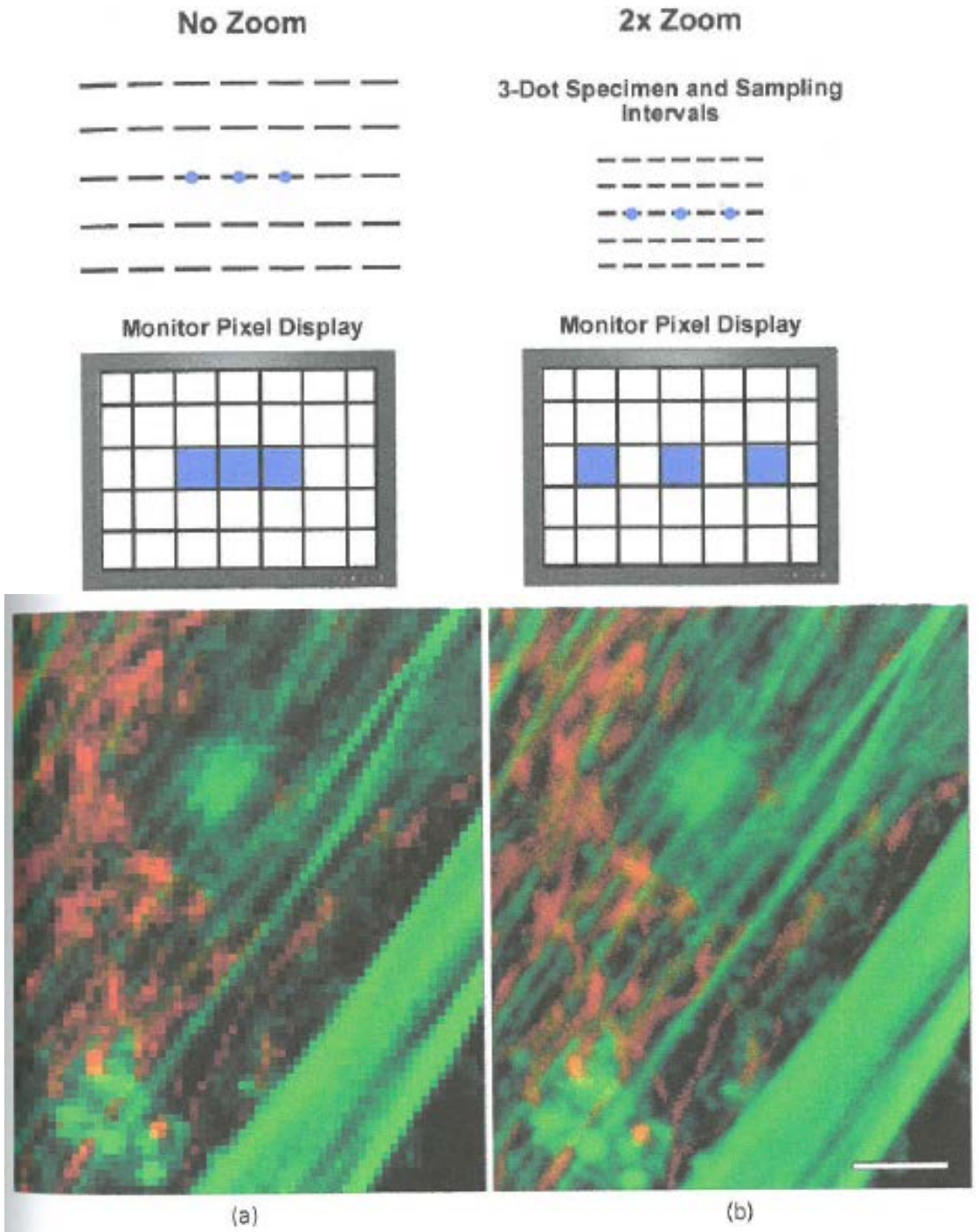






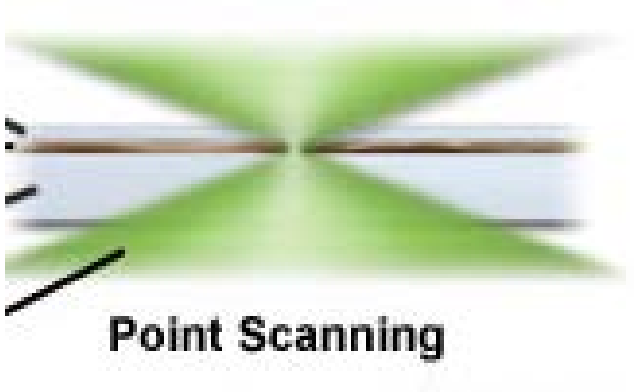
Digital zoom

- Doesn’t make sense to sample at pixels < Nyquist frequency of your diffraction limit
- You can increase resolution until this limit
- Zoom in confocal is set by how far your mirrors travel, and how many times you digitize the signal
- Higher zooms -> greater photobleaching
- Often in the software, you can set an “optimal” zoom



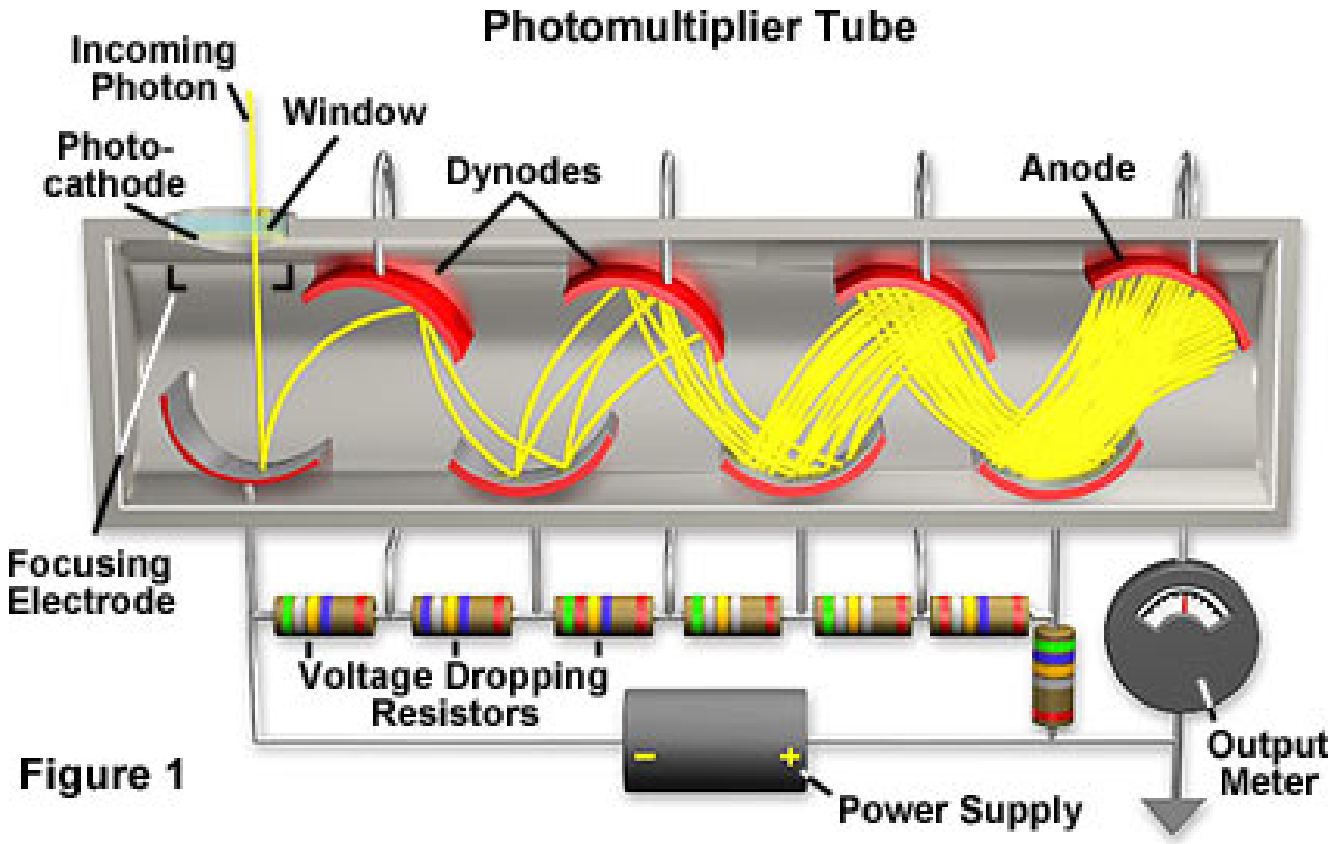
Confocal experimental parameters

- Magnification can be adjusted by varying the area scanned by the mirrors. You don't have to change the objective
- Fewer restrictions on the objective, but they have to be color corrected, and you need to make sure your image can fit into the max FOV
- Photobleaching occurs at all planes, not just the one you're currently imaging
- ~50-100 photons/pixel yield a moderately bright confocal signal, can give SNR of around 20
- Smaller frames -> higher time resolution



Introduction to photomultiplier tubes

- Very sensitive, single element detector of photons
- Unlike a camera with many pixels, PMTs have a single active element
- The magic occurs by converting photons to electrons, which can then be amplified



+s and –s of PMTs

- Very sensitive detectors
- High bandwidth (response within nanoseconds, much faster than cameras)
- Nonlinear gain with voltage
- Difficult to quantify
- Necessarily a single element detector

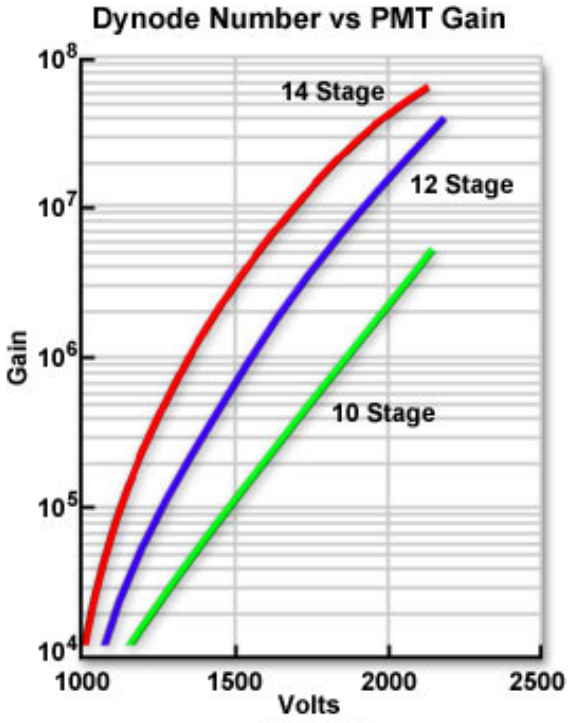


Figure 1

This nonlinear gain makes it hard to utilize the full dynamic range of the sensor

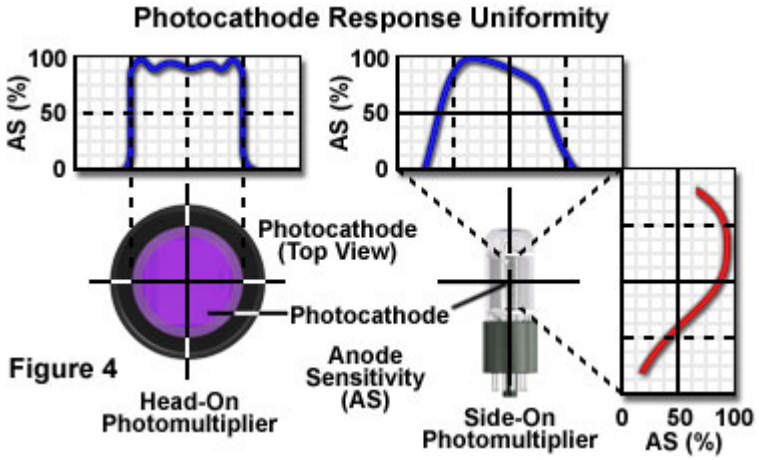
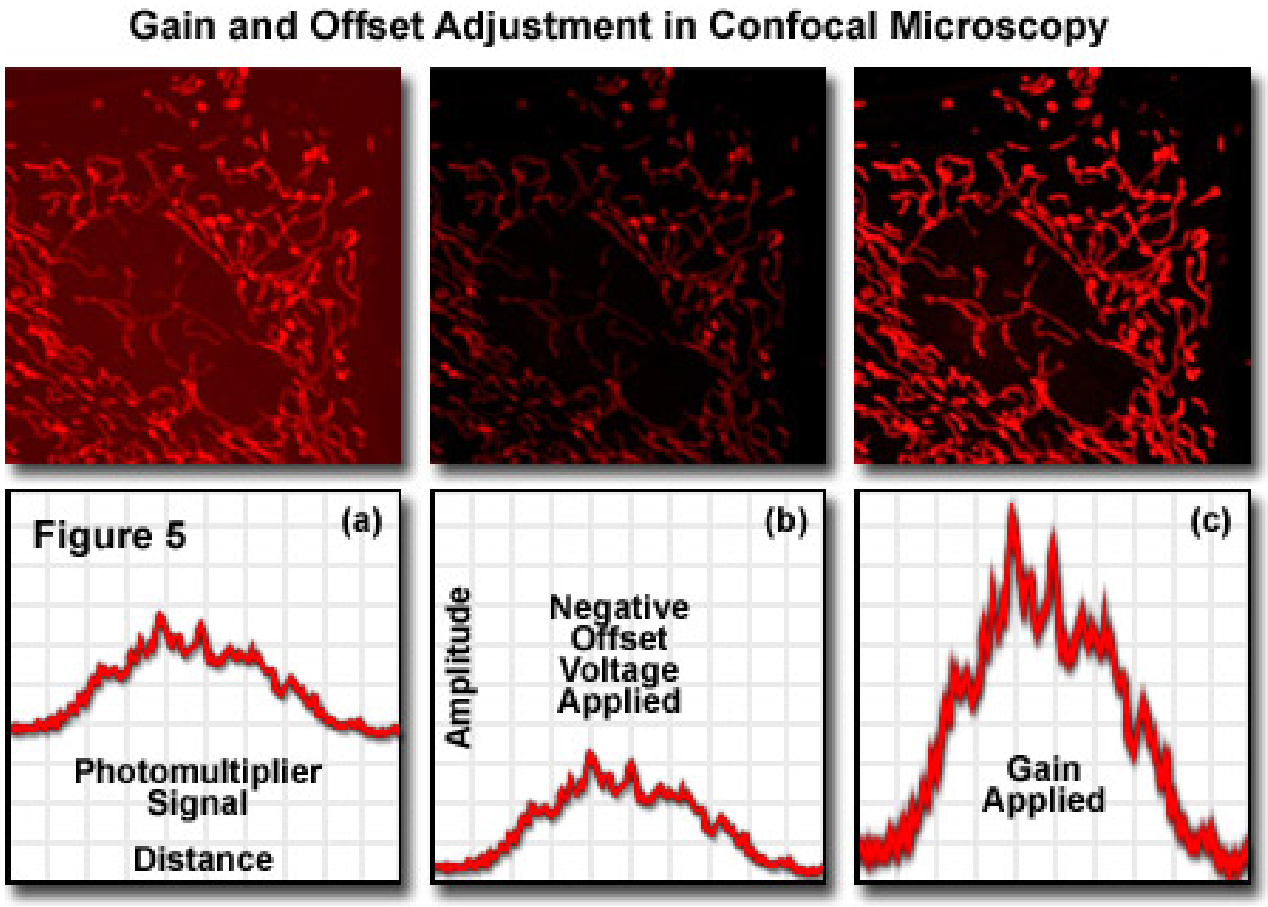


Figure 4

Not completely uniform in their spatial response

Practical adjustments of the PMT

- Record a first image.
- Adjust offset to set background to zero counts
- Add gain to occupy ~90% of saturation
- Inverse relationship between signal and acquisition speed



Hyperspectral imaging

Spectral detection, who needs filters

- Allows for arbitrary color detection at that pixel.
- Color selection is set by position and width of slits.

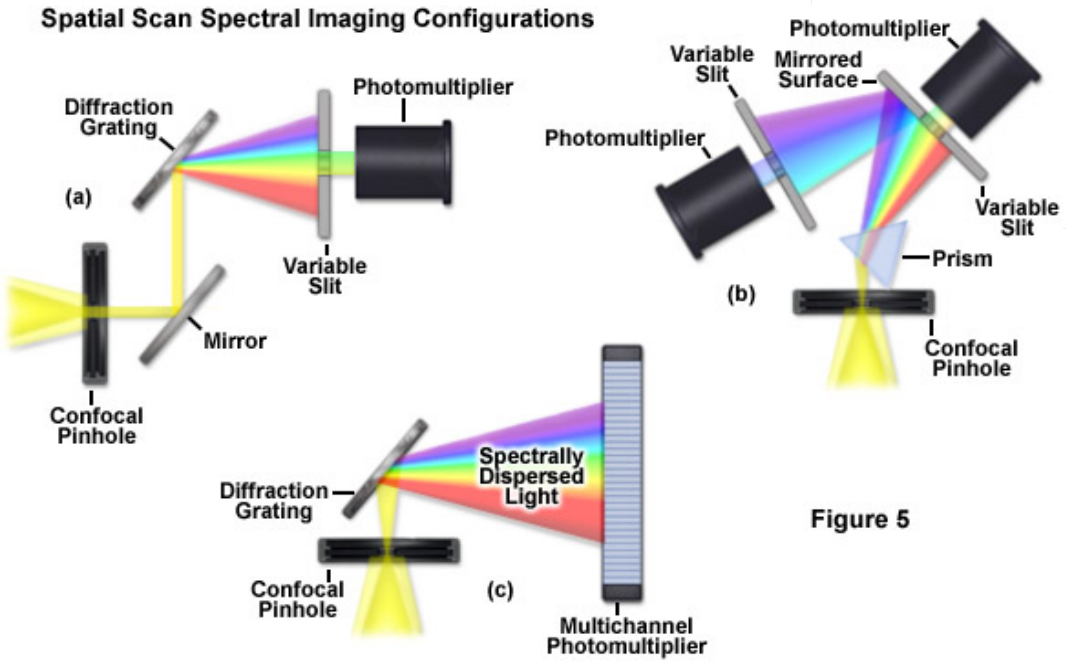
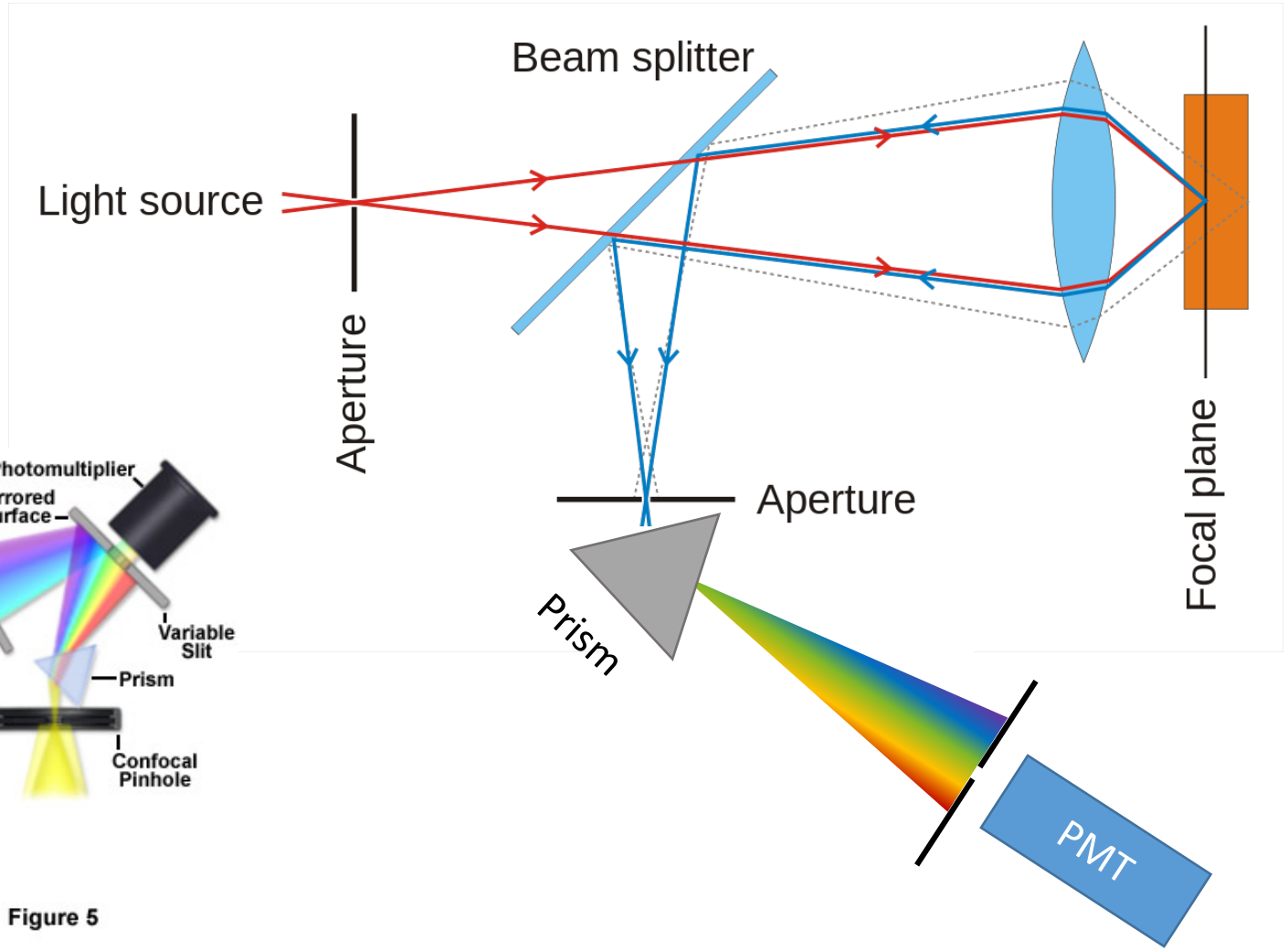
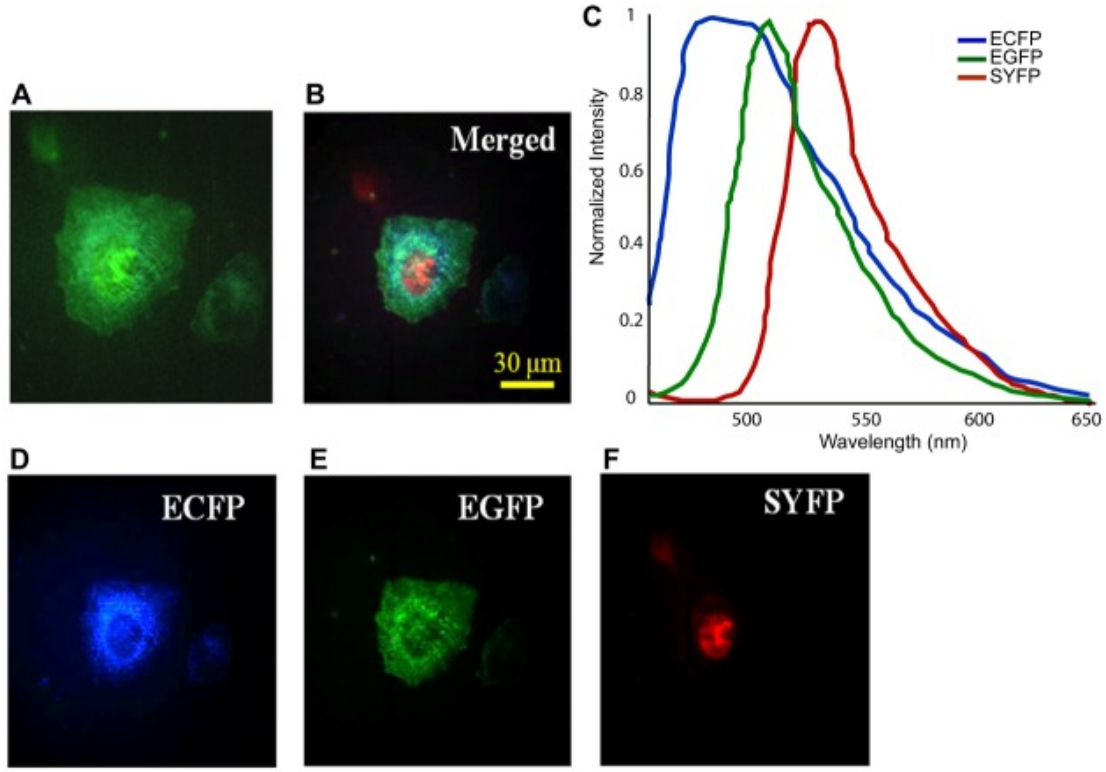


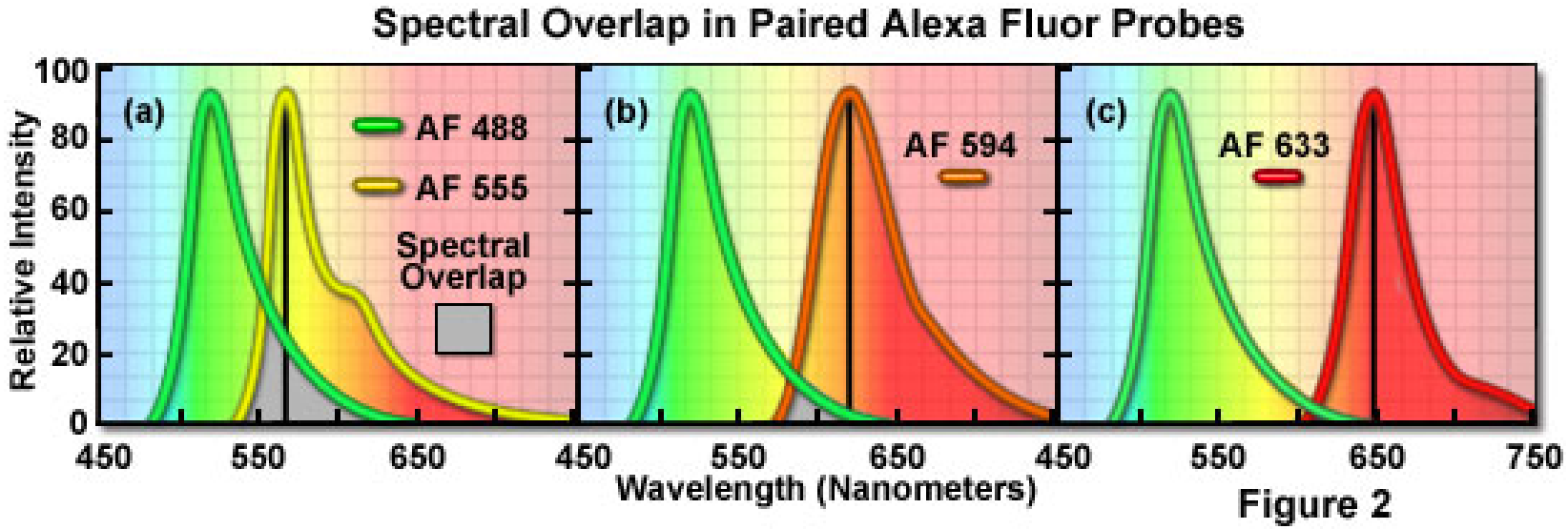
Figure 5



Hyperspectral microscopy

- Compensation for overlapping emission spectra
- At each point, collect a emission spectrum
- Deconvolve the intensity and species of each fluorophore

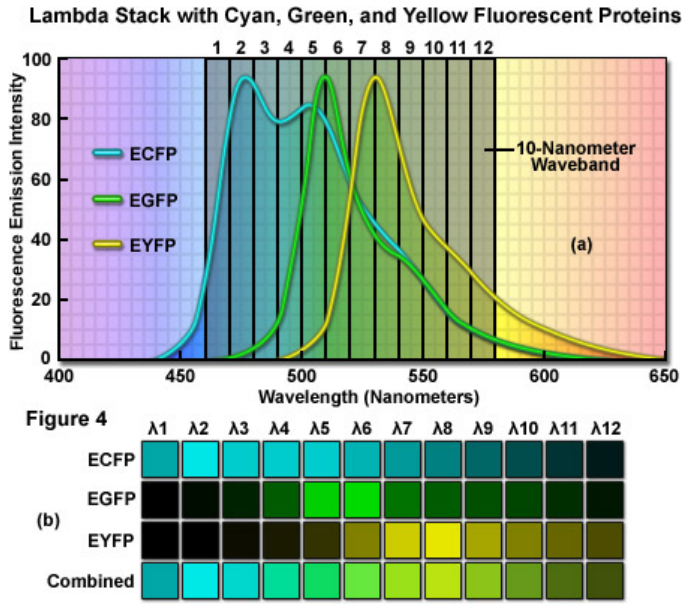


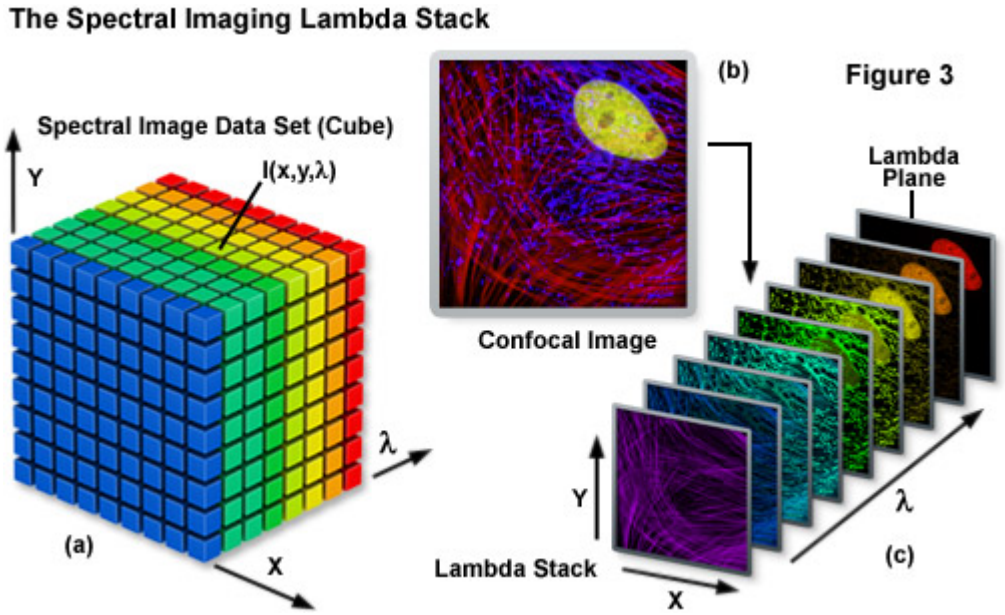


Measuring spectra at each point

- Need to record intensity at each color, at each pixel

$$Intenstity = I(x, y, \lambda)$$





$$S(\lambda) = A_1 * R_1(\lambda) + A_2 * R_2(\lambda) + \cdots A_N * R_N(\lambda)$$

A = Weighting factor
R = spectrum of individual fluorophore

Many software packages will use a linear algebra matrix unmixing to minimize the least squares fit

Spectral unmixing

- We have to assume that the intensity of each fluorophore at each pixel is linear in concentration
- If there are N different species you want to detect, you need to measure L>=N different wavelengths
- Assuming you can measure each fluorophore independently in each channel before you start, it's just a linear algebra problem
- If you can't measure spectra, you can use principal components analysis to estimate number and concentration of species

Consider 3 different fluorophore colors to start, RGB.

We need at least 3 different wavelength measurements.

At each pixel, you record 3 intensitites

$$I(\lambda) = I_R, I_G, I_B$$

The intensities are going to be proportional to how many fluorophores, and how much bleed through there is for each channel.

We can measure the *Smear Matrix*

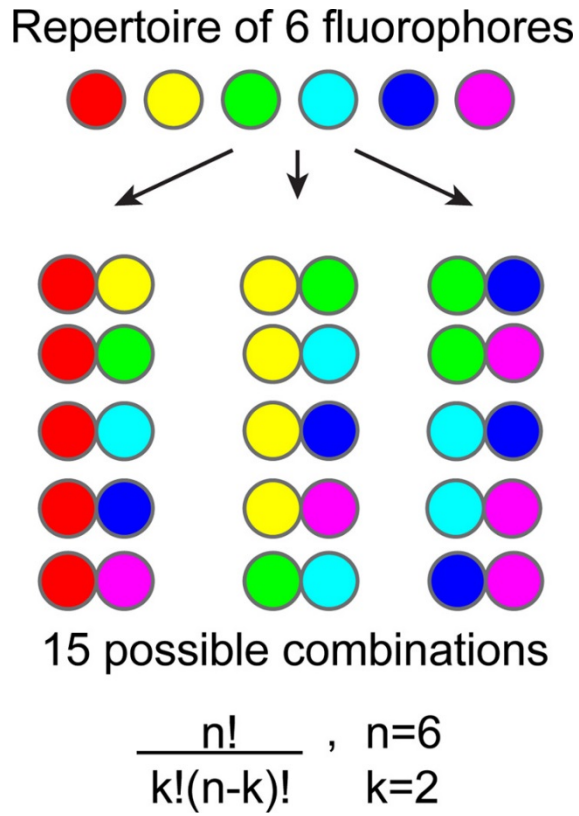
$$\begin{matrix} s_{r,r} & s_{r,g} & s_{r,b} \\ s_{g,r} & s_{g,g} & s_{g,b} \\ s_{b,r} & s_{b,g} & s_{b,b} \end{matrix}$$

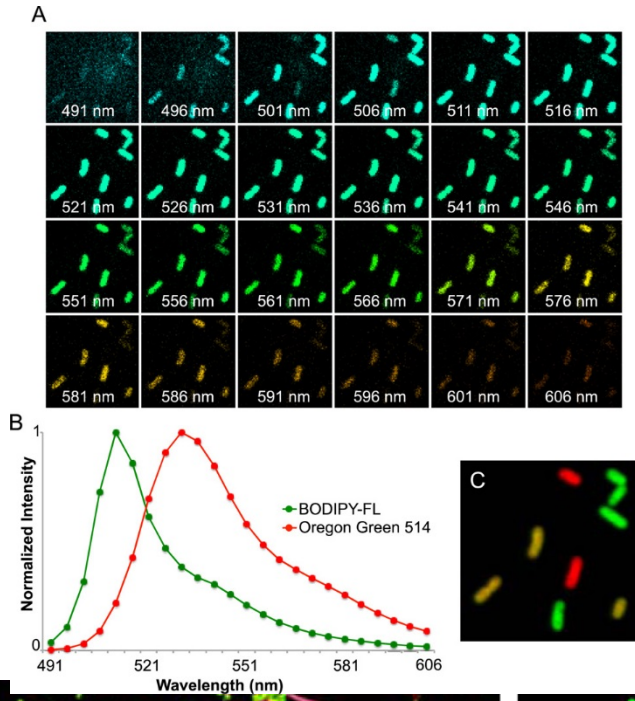
The intensity at each pixel can then be calculated by multiplying the smear matrix by the concentrations

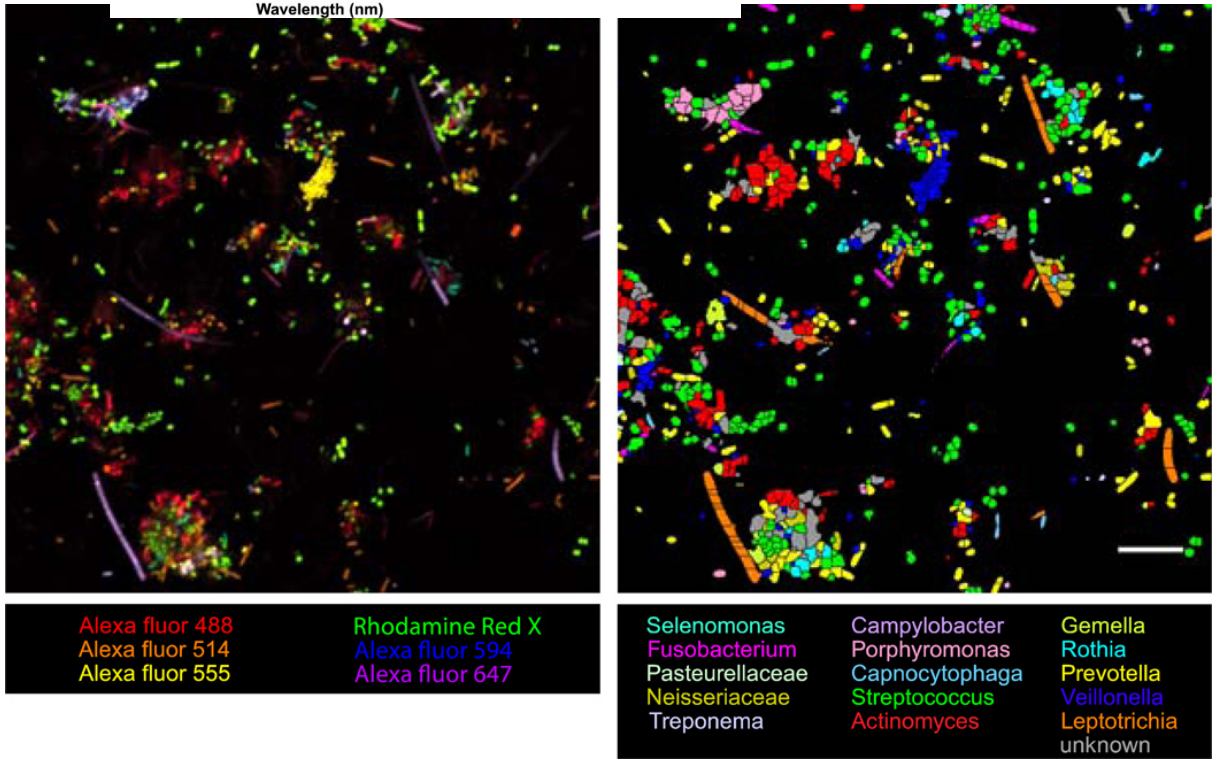
$$\begin{bmatrix} I_R \\ I_G \\ I_B \end{bmatrix} = \begin{bmatrix} s_{r,r} & s_{r,g} & s_{r,b} \\ s_{g,r} & s_{g,g} & s_{g,b} \\ s_{b,r} & s_{b,g} & s_{b,b} \end{bmatrix} x \begin{bmatrix} C_r \\ C_g \\ C_b \end{bmatrix}$$

More spectral unmixing

- CLASI-FISH – distinguish many species of bacteria in a field of view using combinatorial labeling

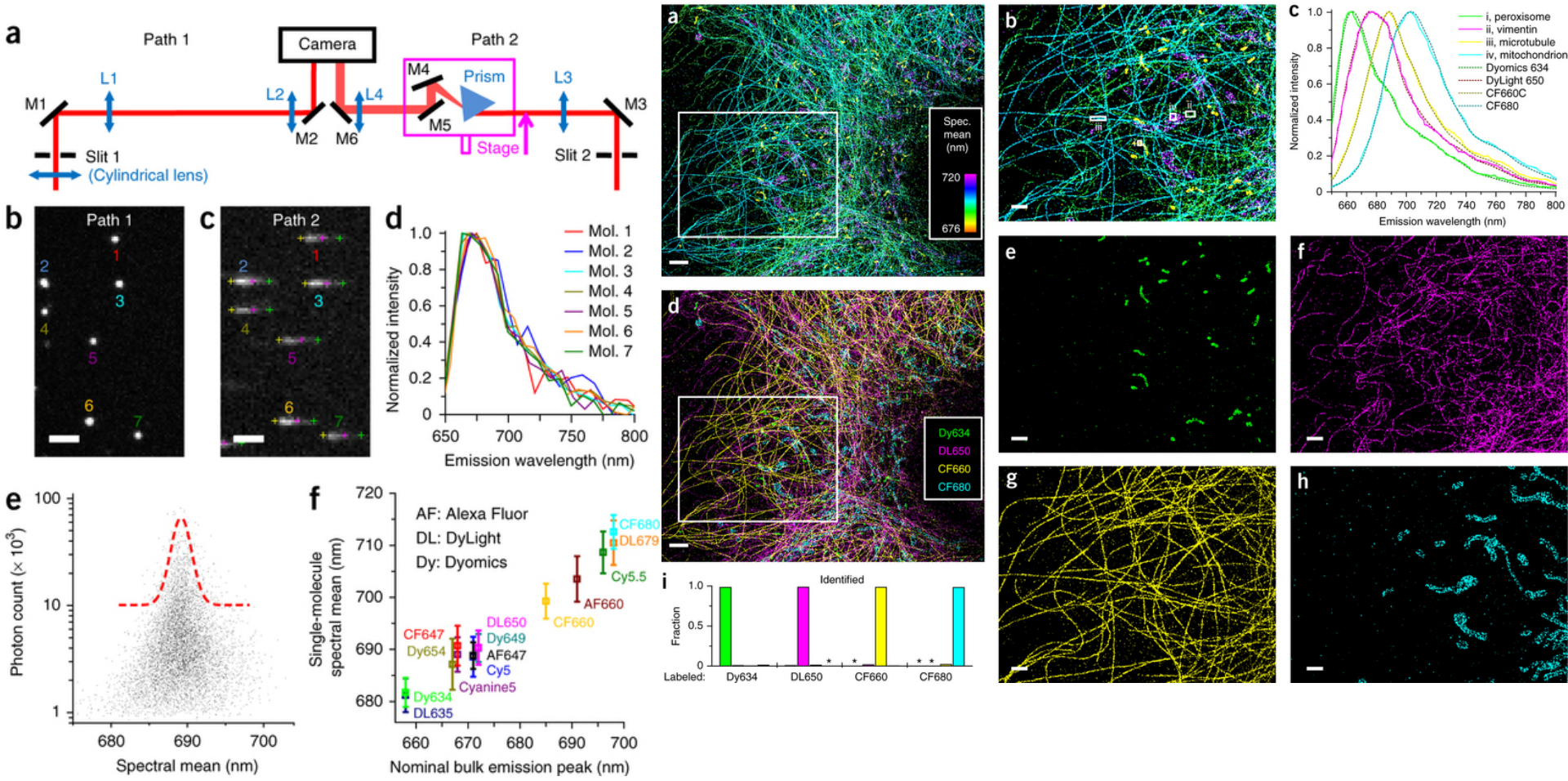






Single molecule spectra

- Taking spectra of single molecules in cells
- Use 4 similar dyes, but unmix their spectra



Spinning disk – speeding up confocal

Fast confocal imaging

- Illuminate many spots on the sample
- Collect emission through many pinholes
- Image onto a camera instead of PMT
- Collect thousands of pinholes simultaneously
- Each frame illuminates entire FOV, so you shouldn't see individual pinholes

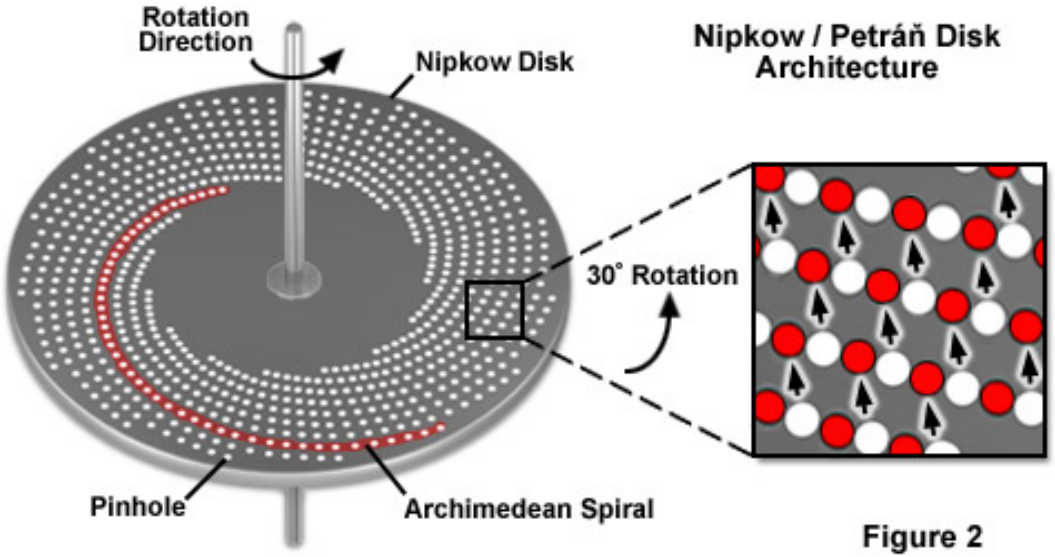


Figure 2

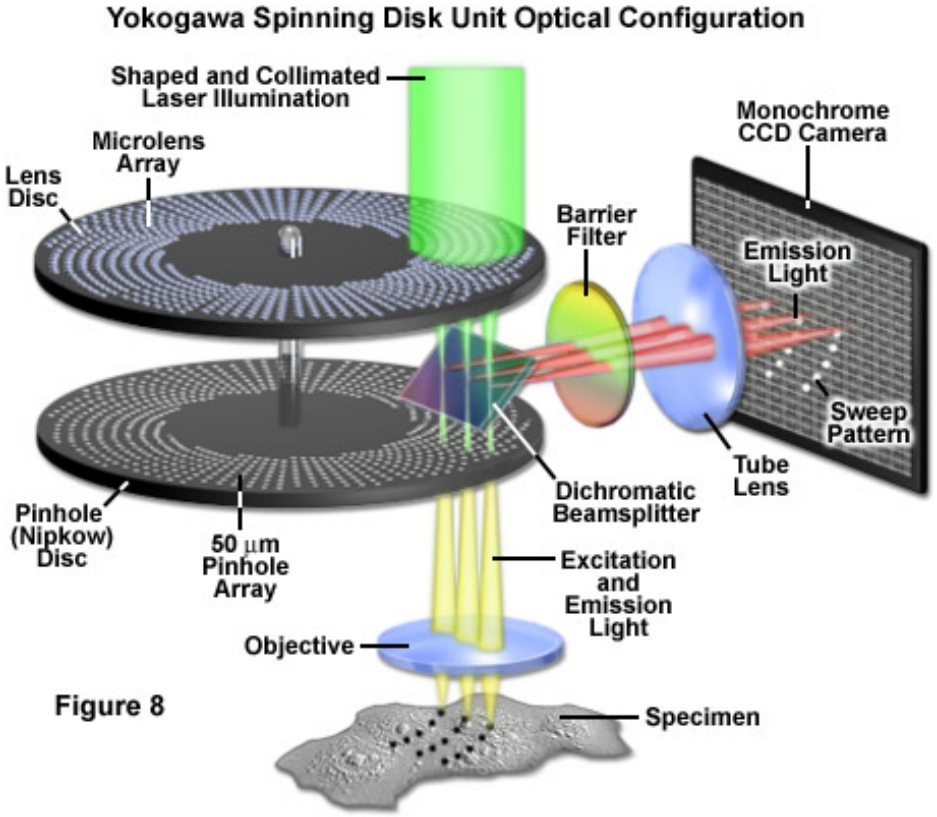
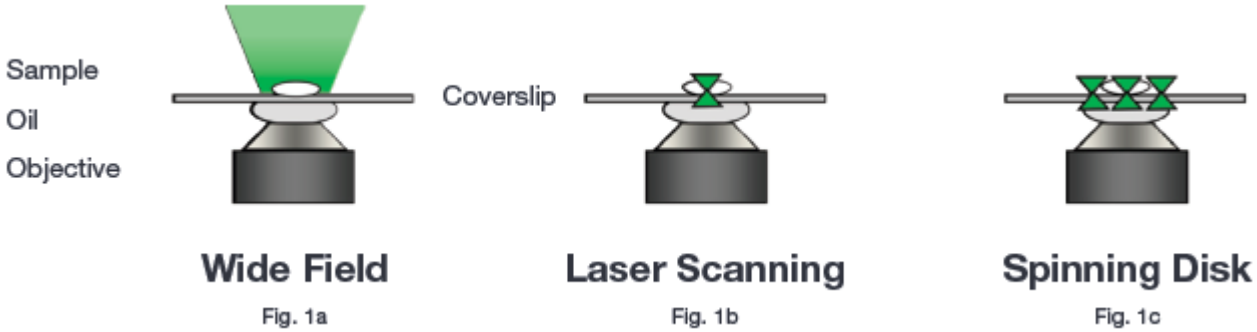


Figure 8



Advantages of spinning disk

- Faster and easier to use than line scan microscope
- Can record up to 1 kHz frame rates (2 Hz at the very fastest for line scan)
- Quantification is easier with a CCD camera
- Lower overall light exposure, lower phototoxicity

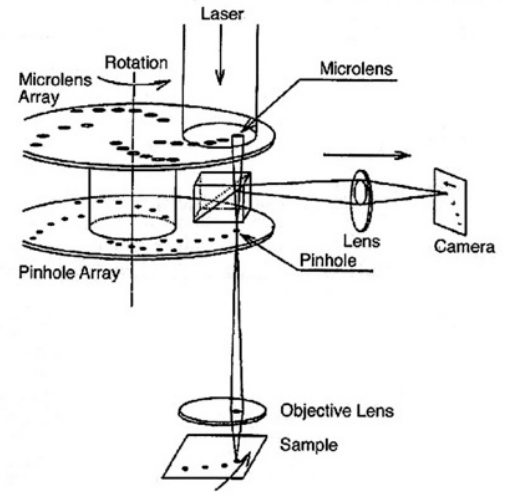
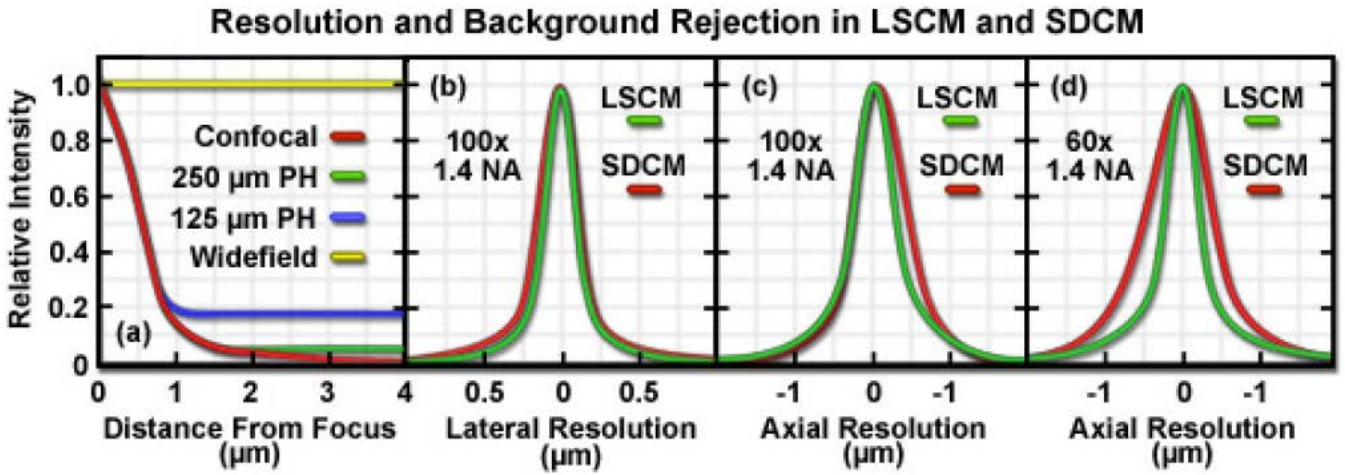


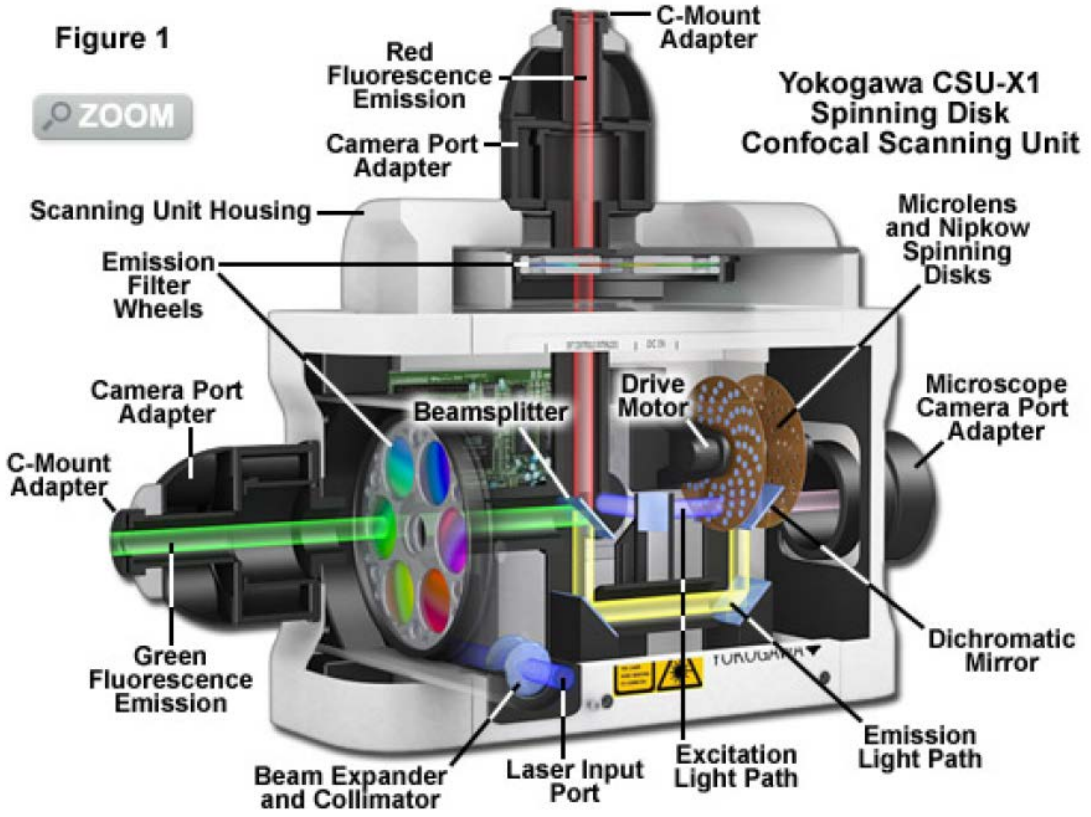
FIGURE 2-71. Yokogawa high-speed confocal system. Microlenses on a second Nipkow disk increase disk transmission to 40–60% instead of a fraction of a percent as in conventional, single-Nipkow-disk systems. The microlens and pinhole arrays are patterned to give a homogeneous field with no sign of scan lines. (From Ichihara *et al.*, 1996.)

"Video Microscopy", Inoue and Spring, 1997



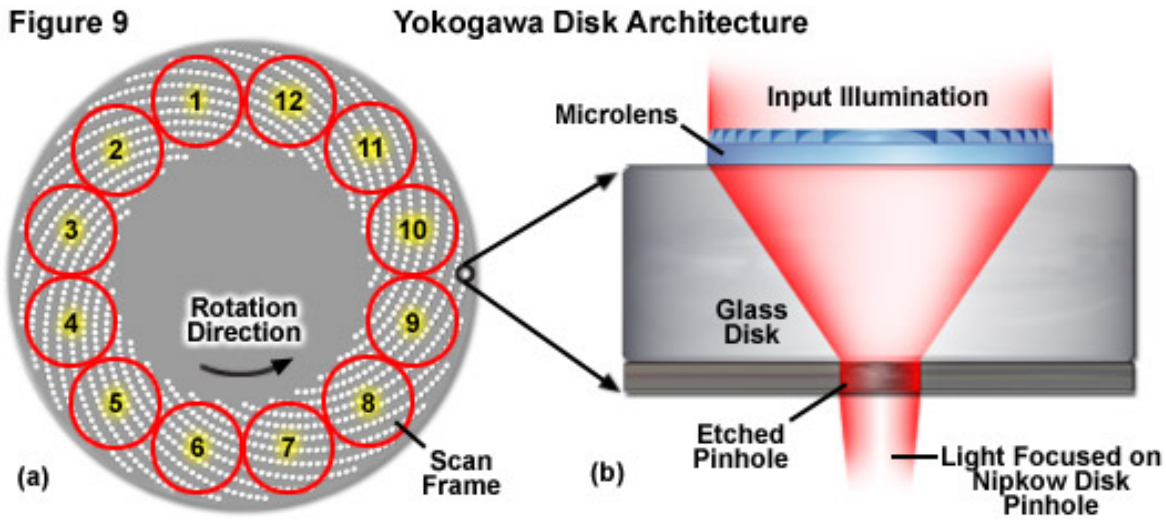
Disadvantages of spinning disk

- Light can travel through adjacent pinholes, cross talk
- Pinhole size is fixed even if you change objectives
- Low level of light transmission through the pinhole, makes it tough for dim samples
- Most excitation light is blocked by disk
- Excitation light travels through dichroic filter
- EXPENSIVE!



Yokagawa disks

- Very little light is coupled through ordinary disk
- Yokagawa uses microlenses on one side of the disk to focus light into pinhole
- Drastically increases excitation intensity



Nested spirals are designed so that 30 degrees will illuminate entire image
12 full images per disk
Fastest disks rotate at 10,000 RPM

-> 2000 frames per second
500 μs per exposure, minimum

And on to Matlab…