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Single Molecule Localisation Microscopy

The past several decades has seen the development of a number of techniques designed to overcome the diffraction limited spatial resolution of microscopes, typically around 250 nanometers for visible light. These techniques are classified as Super Resolution Microscopy.

Single Molecule Localization Microscopy is a wide field sub-set of Super Resolution techniques based on imaging individual molecules many times and then combining these images to obtain resolution as much as 10 times below the diffraction limit.

Light from each molecule is localized on a high sensitivity camera, producing an extended disk at the diffraction limit of the microscope. The disk is analyzed by fitting it in two dimensions with a point-spread-function, typically a Gaussian, describing the diffraction limited signal from a point source. The centroid of the disk provides an estimate of the location of the molecule. The precision of the centroid improves roughly as the square-root of the number of photons imaged.

For example, the precision of the localization of a molecule imaged with 100 photons would be 10 times better than the diffraction limit. The number of photons can be increased by detecting more photons in a given image and/or by adding multiple images. However, it is critical that each molecule imaged is clearly separated from neighboring molecules; otherwise a diffraction limited image will result.

Photek recommends

- **Camera**
  iCMOS 160
- **Software**
  Photek Image32
- **Plug-in**
  University of Sussex
  Single-Molecule ImageJ

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Two common forms of Single Molecule Localization Microscopy are **Photo-Activated Localization Microscopy (PALM)** and **Stochastic Optical Reconstruction Microscopy (STORM)**. The Single Molecules being imaged are tagged with a photo-activated fluorescent dye engineered to fluoresce only after activation by a specific wavelength of light. Once activated, the dyes fluoresce when stimulated by light at a different wavelength.

The number of florescent dyes activated is controlled by the parameters of the activation laser, ensuring that the activated dyes are well separated in the image. Eventually the activated dyes are either deactivated by yet another laser pulse, or turn off due to photo-bleaching. Another set of fluorescent dyes can then be then activated and imaged. A full super-resolution image is obtained by repeating this process many times, localizing each molecule using the centroid technique.

To obtain a single super-resolution image can require tens of thousands of images, each obtained with high sensitivity. The ideal camera for this application is a CMOS based camera like the Photek iCMOS 160. This camera has single photon sensitivity and can operate at high speeds, drastically reducing the time needed to form a Super Resolution image.

Increasing the speed of the camera further through binning and region of interests can enable Super Resolution movies of single molecule dynamics. When coupled with depth sensitive techniques such as light sheet illumination, 4/5D super resolution microscopy can be achieved.

Instrumentation

Below is an example of a typical PALM configuration.

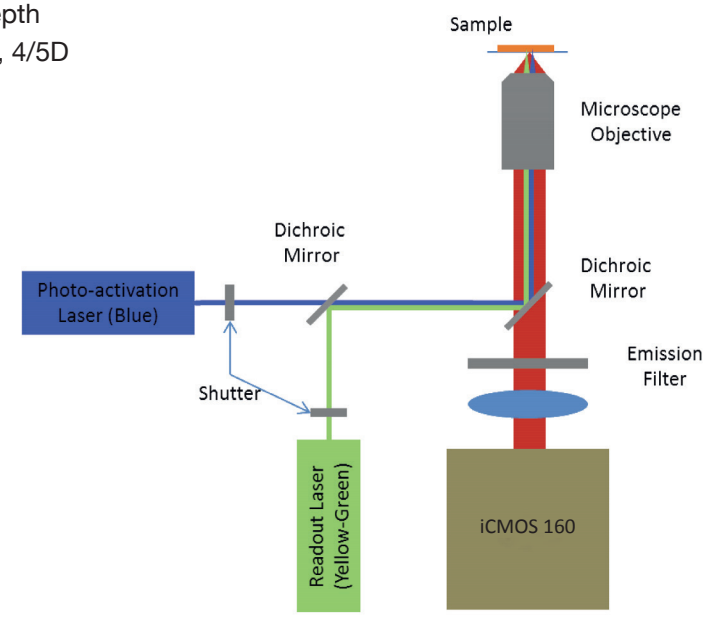
The fluorescent dyes are photo-activated by a blue laser, imaged onto the sample through a series of dichroic mirrors.

A shutter is used to de-select the blue laser and enable a yellow-green readout laser to be focussed onto the sample, stimulated the activated dyes to fluorescence.

The red fluorescence light is passed through a dichroic mirror, filtered to remove extraneous light and imaged onto an iCMOS 160 camera.

After photo-bleaching has deactivated some of the fluorescent dyes, the activation laser is used to activate additional dyes.

This process is continued until sufficient signal has been obtained to reconstruct a super resolution image.



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