Towards 3-Dimensional and time sequenced (4D) live cell imaging

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Outline

• Introduction

• Current State-of-the-Art, concentrating on:
  – Conventional and Fluorescence microscopy
  – Confocal and Multi-Photon microscopy

• Our method
  – Multi-layer imaging by use of a DOE
  – Application to bio-imaging

• Experimental Details
  – Imaging at the nano-scale and practical particle tracking
  – Experimental data using nano-holes and nano-spheres

• The Future
  – Extension to 3D Phase Contrast Imaging
  – Inclusion of Wavefront Sensing
Goals in Biomedical Imaging Microscopy

• Dynamic processes of proteins & molecules
• Imaging of DNA (small scale)
• Virus tracking

• 4D problem (3D + time)
• Nanometer accuracy required

Listeria invading cells
http://ls.berkeley.edu/bio/gallery_mcb/tails.html
Important Properties of the Microscope

Resolution

Successful Imaging

Magnification

Contrast

**Resolution** – The least separation between two separate points at which they are distinguished as separate.

This should be chosen to capture the finest specimen detail.
Important Properties of the Microscope

Resolution

Successful Imaging

Magnification — Enlarging the image sufficiently for it to be accurately sampled by the eye/camera.

Contrast

Intensity $\propto M^{-2}$
Important Properties of the Microscope

Resolution

Successful Imaging

Magnification

Contrast

Contrast – The ability to distinguish the imaged object from other objects and the background.

Infinite resolution without contrast is useless.

Idea taken from SUPA Biophotonics lecture by John Girkin
Requirements for Live-Cell Imaging

Primary Considerations:

• Specimen viability (and consideration of the specimen’s natural environment)
• Signal to Noise (SNR)
• Required Speed/Time Window (to image dynamic processes)

Ideally we want highly sensitive imaging, fast capture rates, high resolution and a wide dynamic range.
Fluorescence

- A large number of bioimaging applications use auto or induced fluorescence.
- GFP and its colour-shifted genetic derivatives allows multicolour imaging.
- Issues: Photobleaching, Fluorophore saturation, pH.

_Aequorea Victoria_ the bioluminescent jellyfish that gave us GFP

_Human Lung Tissue_
Imaging in 3D – Achieving Depth Resolution

• To build 3D images we need:
  
  – To be able to distinguish the signal coming from our plane of interest (focal plane) and the background.
  
  – To image planes at various depths within the sample (called optical sectioning).

Confocal and Multi-Photon Microscopy both achieve this and are widely used as tools for live-cell imaging.
Confocal Microscopy

- Spatial filtering eliminates/reduces secondary fluorescence.

- LSCM
  - Scan the sample or beam to build a 2D slice.
  - Collect serial z images to build the 3D image.

Comparison of a widefield and confocal image of the same neonatal rat neuron

http://www.wadsworth.org/cores/alm/gallery.htm
Multiple Beam Confocal Microscopy

Spinning Disc systems:

- Numerous apertures illuminate hundreds of spots simultaneously.
- Faster scan rates (video rate)
- Higher transmission
- Larger pinholes/slits = thicker optical sections
- Rapid live-cell imaging at reasonable cost.

Image from www.microscopyu.com
Confocal Microscopy – Pro’s and Con’s

Advantages:

• The ability to produce thin optical sections.
• Increased SNR (reduction of background fluorescence).
• Zoom factor – adjust spatial resolution by altering the scanning laser sampling period.
• Non-invasive, allows examination of live and fixed specimens.

Disadvantages:

• Limited number of excitation wavelengths (laser lines) available.
• Scanning speed limits data acquisition rates.
• Photodamage risks with high powered lasers.
• Time delay in obtaining 3D images.
Multi-Photon Microscopy

• Two photons arrive simultaneously and combine their energies to excite the fluorophor.

• NIR excitation wavelengths mean:
  – Higher depth penetration
  – Less scattering
  – Reduced photo-damage
Comparison of one-photon and two-photon imaging

http://www.bris.ac.uk/Depts/Anatomy/research/neuro/OneTwoPhoton/TwoPhoton.htm
Multi-Photon Microscopy

Key Property:

- Excitation confined almost exclusively to the focal plane (no pinholes required, increased efficiency, greatly reduced photo-damage).

- Lasers – High peak power (ultrashort pulses), low average power. Single photon excitation and heating effects are reduced.

www.microscopyu.com
Multi-Photon Microscopy

This image demonstrates that one-photon excitation creates fluorescence throughout the focal illumination cone. Two-photon on the other hand only excites fluorophors at the exact focus.

http://www.phys.ntu.edu.tw/biophys/English/EPTM.htm
Multi-photon Microscopy – Pro’s and Con’s

Advantages:

- Uses longer ‘friendlier’ wavelengths.
- Deeper penetration, less scattering.
- No pinholes, greater efficiency.
- Photobleaching and photo-damage minimised, and localised to the focal region.
- Dramatically increased SNR.

Disadvantages:

- Significant work still required to profile common fluorophors and develop new ones for multi-photon work.
- Time delay in obtaining 3D images.
- Complexity and cost.
Proposing a new method....

We have used a specially designed DOE to:

- Provide snapshot imaging of multiple object planes simultaneously onto a single image plane.

- Applications: $M^2$ measurements, wavefront sensing, particle tracking, and now *bioimaging*

*So how does it work?*
D.O.E’s – The Basics

• In its simplest form a diffraction grating is a grid of multiple slits.
  – Amplitude grating ~ formed by alternate opaque/transparent regions
  – Phase grating ~ variation in optical thickness provides a phase delay

• Transmitted beams interfere to produce the familiar alternating pattern of maxima and minima.
Imaging with a straight grating…

The lens equation:

\[
\frac{1}{f} = \frac{1}{u} + \frac{1}{v}
\]
Detour Phase

By ‘bending’ the straight rulings the grating we can change its imaging properties.

• Detour phase is the phase shift added to each diffraction order by the distortion.

Distorting the diffraction grating to produce a quadratic (defocus) detour phase results in an important property:

The focal length in each diffraction order is different
Properties of the QD grating

- The focal length in each diffraction order is determined by the amount of detour phase added.

- The grating is used as a tool to artificially propagate the plane which is in focus in the $0^{th}$ order by $\pm f_G$ in the $\pm 1$ orders, $\pm 2f_G$ in the $\pm 1$ orders.....

- Can be set up to capture images about the focal plane of the lens or about a pupil plane.

- Dynamic range largely determined by the distance between the imaged planes (i.e. $f_G$)
Multi-Plane Imaging with a QD grating...

\[ f_G = \frac{R^2}{2mW_{20}} \]

\[ f_c = \frac{f_L f_G}{f_L + f_G - S} \]
Imaging at the nano-scale

• Moving from imaging on the macroscopic scale to the nano-scale is not a trivial problem!

• The system must now incorporate:
  – high magnification
  – high-precision positioning control
  – vibration isolation
  – make every photon count!

Samples:
  – Nano-holes and Nano-spheres (210nm).
  – Simulate fluorescently tagged bio-particles.
  – Chosen for their size and point source behaviour
The Microscope

Image of one nanohole

CCD

f = 200mm

Magnified Image

iris

∞ corrected Objective

100x NA 1.3

Fibre illumination (633nm)

Piezo Positioners (x,y,z)

Nanoholes
Through focal (z) series
Measuring Performance

Measure the FWHM and compare to the diffraction limit:

**Single image cross section**

**In focus image**

**Sparrow’s Limit = 244nm**
Adding the grating…

Imaging on 3 object planes

CCD

QD DOE

f_c = 215mm

f = 200mm

iris

Piezo Positioners (x,y,z)

100x NA 1.3

Fibre illumination (633nm)

Towards 4D Live Cell Imaging
Imaging on 3 planes

-1 order in focus

0th order in focus

+1 order in focus

Measured Resolution:

Without grating = 233nm
With grating = 226nm and 231nm (for 0th and ±1 orders respectively)
Practical Particle Tracking

- **Aim:** to track particles in real-time in 3 dimensions (4D).
- A Centre Of Mass calculation on each intensity image provides the x,y co-ordinate.
- We have developed a method of determining the position in z using the concept of *Image Sharpness*.

- For another application of this system to Particle Imaging Velocimetry see:

Image Sharpness

Image Sharpness is given by integrating the normalised MTF plot.
Calculating Image Sharpness

1. Take the Fourier Transform of the image.
2. Take the modulus of the Fourier Transform to obtain the OTF.
3. Integrate the OTF in 2D to obtain the MTF.
4. Normalise the MTF to obtain the normalised MTF.
5. The normalised MTF represents the image sharpness.
Image Sharpness –
A Single Focal Series

- Ambiguity in $z$ when using only a measure of the Image Sharpness.

Our Solution?

Compare the Sharpness curves for two (or more) different orders.
Image Sharpness Plots (±1 orders)
Modelling the Sharpness

• Simulated data is created to match the experimental data as closely as possible using:
  – The defocus added by the grating.
  – The additional defocus due to the movement of the source.
  – Linear relationship between the Spherical Aberration (SA) in the system and the defocus from the source movement.

N.B. Simulated data is noiseless and contains less aberrations.

• The Image Sharpness is then calculated in exactly the same way, but this time using the simulated data.
Modelling the Sharpness

![Graph showing experiment vs model comparison in sharpness vs distance (micrometres)]
Accuracy in z
~ Measuring the Ratio
Accuracy

Initial analysis to assess the accuracy of the position measurement:

• Accuracy in x,y (COM)
  – 50 snapshots taken at each position.
  – Standard deviation gives repeatability error of better than 40nm.

• Accuracy in z (Sharpness measurement)
  – Sub-nanometer over the central linear region.
  – Region of maximum accuracy could be increased by decreasing the depth of focus.
The Quest for Brownian Motion

- **Aim:** To study single particles moving under Brownian motion.

- Fluorescent nanospheres used to simulate moving bio-particles.

- **Challenge:** To maximise use of the available light (without bleaching) and with fast exposure times.

Seisenberger *et al* Science 294 p1929 (2001)
Tracking a moving particle…
Transmitted Light Contrast Imaging

• Transmitted light techniques are often used to study cell shape, position and motility (i.e. during mitosis).

• Intensity contrast is achieved in unstained specimens by manipulating/removing the un-diffracted portion of the transmitted light.
Achieving Contrast – Manipulating the Fourier Plane…

Red = light scattered by sample
Black = un-diffracted beam

Schlieren – Knife edge
Dark Field – Occulting Spot

Phase Contrast – λ/4 Phase Spot
3D Phase Contrast Imaging...

- CCD
- QD DOE
- f_c = 215mm
- f = 200mm
- iris
- Magnified Image
- 100x NA 1.3
- Phase Spot
- Piezo Positioners (x,y,z)
Initial Results - Schlieren Imaging

Results taken by Carola Diez

Contrast improves as the knife edge is stepped further into the focal spot.
Extension to Wavefront Sensing

The shape and phase of the input wavefront can be calculated using the same intensity images we use to perform the particle tracking.

The difference of the ±1 order images is an approx. of the axial intensity gradient.

Options for phase retrieval:
- Iterative solution (Gerchberg-Saxton, Simulated Annealing etc)
- Greens function (reported accuracy $\lambda/1000$)
- Analytically using the SAE (still in development).
Wavefront Sensing

- Specimen-induced aberrations reduce signal levels and cause image degradation.

- Wavefront Sensing would allow:
  - Characterisation of system aberrations.
  - Characterisation of specimen-induced aberrations.

- Possibility of aberration correction (AO) or pre-compensation (in the grating).

For more information about the problems caused by specimen-induced aberrations see:
References

Live-Cell Imaging:
- Stephens, D.J. and V.J. Allan, "Light Microscopy Techniques for Live Cell Imaging". Science. Vol. 300, p. 82-86. (2003) [N.B. this was a special issue containing several interesting papers on bio-imaging]

Microscopy Resources:

Websites:
- www.microscopyu.com [this is a very extensive source of information on all aspects of microscopy!]

References (cont…)

The Quadratically Distorted (QD) grating multi-plane imaging method:

Wavefront sensing with the QD grating:

Particle tracking with the QD grating:
Please visit our website:

This presentation is available to download from the Waves and Fields group website:

http://waf.eps.hw.ac.uk