Optically sectioned imaging by oblique plane microscopy

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Abstract: This paper describes a new optically sectioning microscopy technique based on oblique selective plane illumination combined with oblique imaging. This method differs from previous selective plane illumination techniques as the same high numerical aperture lens is used to both illuminate and image the specimen. Initial results obtained using fluorescent pollen grains are presented, together with a measurement of the resolution of the system and an analysis of the potential performance of future systems. Since only the plane of the specimen that is being imaged is illuminated, this technique is particularly suited to time-lapse 3-D imaging of sensitive biological systems where photobleaching and phototoxicity must be kept to a minimum, and it could also be applied to image microfluidic technology for lab-on-a-chip, cytometry and other applications.

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References and links

1. Introduction

Conventional optical microscopy provides high resolution (~200 nm) images for a huge range of applications, from inspection of electronic devices to cell biology. However, in many situations it is desirable to obtain optically sectioned images, which usually consist of axially thin slices through the sample. The advantages of optically sectioned imaging include improved image contrast through the reduction in contributions from out-of-focus planes and the ability to produce 3D images. The most commonly used method for obtaining high quality optically sectioned images is confocal microscopy, which involves scanning a point of illumination and detecting the reflected or fluorescent light using a confocal point detector [1]. However, the need to scan the point source through the sample in two or three dimensions can limit the achievable imaging speed. While this can be mitigated by rapid beam scanning, the associated high peak excitation beam intensity has the potential to cause increased photobleaching or photodamage to biological samples.

An alternative approach to faster confocal imaging is the use of multiple excitation and detection spots, e.g. in a Nipkow disk microscope [2]. However, there is then a trade-off between the number of excitation beams and the cross-talk between detection pinholes, which can degrade the quality of the optically sectioned image [3]. A number of alternative optical sectioning methods have been proposed that exploit post-processing of multiple wide-field image acquisitions to calculate optically sectioned images, including ‘structured illumination’ techniques, e.g. [4]. With such methods the wide-field illumination means that fluorescence images can be rapidly acquired using low excitation intensities, however, the unwanted out-of-focus light is still detected by the CCD camera and this degrades the signal to noise ratio in the final image compared to ‘true’ optical sectioning techniques. For both confocal and structured illumination techniques the sample is illuminated throughout its whole axial extent for each image acquisition, even when only a single lateral plane in the sample is being imaged. This leads to photobleaching and phototoxic effects in the planes that are not being imaged, which is undesirable when imaging live biological samples.

Another method for performing optically sectioned imaging is that of Fluorescence Coherence Tomography (FCT) [5], which makes use of the phase of the emitted fluorescence to provide axial resolution. This technique requires that the specimen is imaged with two opposing microscope objectives. In the case of spectral-domain FCT, the axial information is calculated from a spectrally resolved interferogram, with the result that the signal to noise ratio in the final image is dependent on the axial distribution of fluorophores within the specimen [6].

Recently, a new technique for obtaining optically sectioned images, described as Selective Plane Illumination Microscopy (SPIM) [7], has been developed, which does enable optically sectioned imaging with only the image plane being illuminated. This technique extends earlier work by Voie et al. [8] and Fuchs et al. [9], and uses two objective lenses orientated at an angle of 90° relative to one another to image the sample, as illustrated in Fig. 1(a). One lens is used to illuminate only a thin ‘sheet’ within the sample and the second lens is used to produce a wide-field diffraction-limited image of the illuminated plane. SPIM thus provides ‘true’ optical sectioning while benefitting from the speed of wide-field image acquisition and causes minimal photobleaching and photodamage to the sample. SPIM has been used to obtain images of small organisms and embryos and can be used to image both reflected or scattered light and fluorescence [7, 10]. It can also be combined with optical clearing techniques to decrease scattering, e.g. [11].

A potential drawback of SPIM is that it requires the sample to be illuminated with a lens that is in the plane of the sample being imaged and this means that conventional sample
preparation techniques, e.g. glass microscope slides, cannot be used. Recent work by Holekamp et al. [12] has addressed this issue to allow ex vivo imaging of live tissue slice preparations using a tilted SPIM-type configuration. However, this technique requires the objective lens to have a sufficiently long working distance such that it does not collide with the specimen, and this limits the NA and therefore resolution that can be achieved.

Recent work by Tokunaga et al. [13] and Konopka et al. [14] has shown that it is possible to illuminate a thin sheet in a sample using the same objective that is used to collect the fluorescence, see fig. 1(b). These imaging systems were termed Highly Inclined Laminated Optical sheet (HILO) microscopy and variable angle epi-fluorescence microscopy respectively. These techniques can be used to acquire a 3D image of the specimen by scanning the sheet illumination or specimen in a single direction. The HILO approach is similar to SPIM but with two significant differences: the illumination and detection beams are not at 90° (as is usual for SPIM) and the sheet of illumination does not align in the focal plane of the imaging system used to collect the reflected/scattered light or fluorescence. This is shown in the image plane of fig. 1(b), where the image of the sample fluorescence (shown as a green stripe) lies at an angle to the image plane (dashed line). The resulting defocus is most severe for regions of the image that are furthest from the centerline of the image and this limits the field of view that can be achieved. The defocus cannot be overcome by simply tilting the detector with respect to the optical axis due to the unwanted spherical aberrations that would arise [15].

The aberrations associated with imaging planes away from the designed focal plane of a microscope have recently been discussed in detail by Botcherby et al. [15, 16] for achieving remote scanning of the imaging plane of a microscope with no movement of the objective lens relative to the sample. They address the issue of defocus by coupling the output from a microscope into the input of a second matched de-magnifying optical ‘microscope’ system. The total magnification provided by these two microscopes is arranged to be equal in the axial and lateral directions. As the intermediate image is not distorted by high axial magnification and the associated spherical aberrations that arise away from the designed image plane, it is then possible to image and re-magnify different planes within the specimen using a third microscope system. This paper shows that a similar concept can be applied to HILO/SPIM, where the third microscope is used to tilt the image plane, rather than using it to image planes located at different axial positions. Thus in-focus imaging of an oblique plane within the specimen is achieved. By taking advantage of the fact that high numerical aperture (NA)
microscope objectives have an angular extent that is much larger than 90°, oblique plane microscopy (OPM) enables an oblique plane in the specimen to be illuminated and imaged with the same objective lens. This technique can therefore be readily implemented on a conventional microscope for imaging specimens prepared using standard glass-slide preparation techniques while retaining the minimal photobleaching and phototoxicity advantages of SPIM. OPM is not restricted to fluorescent samples and may also be applied to imaging with reflected or scattered light. It is anticipated that, with the implementation of more complex detection schemes, it will also provide polarization resolved and spectrally resolved imaging as well as fluorescence lifetime imaging.

2. Method

The concept of OPM was demonstrated using the experimental set-up shown in Fig. 2. Excitation light was provided by an intracavity frequency doubled c.w. Nd:YVO₄ laser at 532 nm with a maximum output power of 1 mW. In order to create an oblique sheet of illumination within the specimen the laser beam was first expanded using a 10× telescope (not shown in Fig. 2). The light was then focused by a cylindrical lens onto mirror M1 and directed to the sample via lens L2, a dichroic mirror (565DCLP, Chroma Technology Corp., VT, USA) and the high numerical aperture objective lens L3. Mirror M1 allowed the angle of the oblique sheet illumination at the specimen to be conveniently adjusted. In this experiment, lens L3 was an UPlanSApo 60×/1.35 oil immersion objective lens (Olympus UK Ltd, UK). The angle of the illumination sheet was set to be at 60° to the optical axis.

Fluorescence from the specimen was then collected by the same objective and an intermediate image plane, IP1, is produced by the first infinity corrected microscope formed by lenses L3 and L4 (f=100 mm, achromat, Edmund Optics Inc., NJ, USA). An emission filter (HQ610/75, Chroma Technology Corp., VT, USA) was used to block unwanted excitation light. Light at image plane IP1 is then de-magnified by the second infinity corrected microscope formed by lenses L5 (f=100 mm, achromat, Edmund Optics Inc., NJ, USA) and L6. Lens L6 was a 40×/0.85 air objective (UPlanApo, Olympus UK Ltd, UK).

In order to re-image any plane within the specimen, it is necessary to ensure that the lateral and axial magnifications between the specimen and FP₆ (Fig. 2) are equal. This condition is achieved when the magnification is equal to n₁/n₂, where n₁ is the refractive index of the immersion medium of the specimen and n₂ is the refractive index of the immersion medium of
lens L6 [15]. Here lens L3 is an oil immersion objective and so the magnification was chosen to be 1.5×. It was then possible to re-image any plane in the specimen using a third microscope system, which was formed here by lenses L7 (10×/0.3 air, Olympus UK Ltd, UK) and L8 (f = 350 mm, achromat, Edmund Optics Inc., NJ, USA). The angle, $\alpha$, between the axes of L6 and L7 was set to be 30°. This results in an oblique image of the specimen with a magnification of 29× in the oblique plane, which was chosen to give a similar magnification to that obtained at IP1 (33×). To help explain the geometry of the oblique plane imaged by the system, a schematic of the focal region is shown in Fig. 3.

Finally, the fluorescent signal was recorded using an analogue CCD camera (PE2015, Pulnix, CA, USA). As this is a relatively low cost (and therefore noisy) CCD camera, all images presented in the results section below are the average of 10 acquired frames. A background frame, again averaged over 10 frames, was also subtracted to remove the constant offset of the CCD readout electronics and fixed-pattern noise. It should be noted that, in the optical arrangement presented in Fig. 2, lenses L3 and L4 could be those already present in a commercially available microscope system, provided the spacing between these lenses is configured as shown in Fig. 2.

The constraints for the angle and angular extents of the excitation and collection beams are shown in Fig. 4. As the illumination and collection beams must be separated by 90°, it is straightforward to derive an expression for the half angle of the collected fluorescence emission, $\phi_{em}$, as a function of the half-angles subtended by lens L3, $\theta$, and the illumination beam, $\phi_{ex}$:

$$\phi_{em} = 2\theta - \phi_{ex} - \pi / 2.$$  

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Fig. 4. Diagram showing the relationship between the illumination and collection angles and numerical apertures: \( \theta \), half angle subtended by lens L3; \( \phi_{ex} \), half angle subtended by excitation light; \( \phi_{em} \), half angle subtended by collected fluorescence emission. The angle between the centres of the excitation and emission ray bundles is \( 90^\circ \).

The numerical aperture of the objective lens is defined as \( NA = n \sin \theta \), where \( n \) is the refractive index of the immersion medium. The full-width at half maximum (FWHM) of the focal spot is given by

\[
d_{\text{FWHM}} = \frac{\lambda}{2n \sin \theta}.
\]  

For the 60×/1.35 objective lens used here, the definition of numerical aperture gives \( \theta \) to be \( 63^\circ \). If \( \phi_{ex} \) is chosen to be \( 3^\circ \), then equation 1 gives \( \phi_{em} \) to be \( 33^\circ \), and so the potential NA of the OPM system is then equal to \( NA_{\text{potential}} = n \sin \phi_{em} \). As an oil immersion objective was used, this angle corresponds to a maximum potential collection NA of 0.83. Unfortunately, the best long working distance objective available to use for lens L7 was a 10×/0.3 objective with an angular acceptance of \( \gamma = 17^\circ \). This limited the actual NA that could be achieved in this prototype system to \( NA_{\text{actual}} = n \sin \gamma = 0.45 \). \( NA_{\text{actual}} \) can be larger than the NA of L7 due to the fact that the specimen is immersed in a medium with a refractive index of 1.5. Put another way, the magnification of the specimen to plane FP6 (fig.2) is 1.5, and so lens L7 is collecting light from an already magnified image of the specimen. The numerical aperture of the lens used for L6 would also have prevented the maximum potential oblique plane collection NA of L3 being achieved; higher NA objectives than those used here for L6 and L7 are commercially available. A collection NA of 0.45 corresponds to a spot with a FWHM of 0.67 \( \mu m \) for a fluorescence emission wavelength of 610 nm, which is therefore the theoretical maximum achievable resolution in the \( x_o \) and \( y_o \) directions (defined in Fig. 3) for the oblique plane microscope demonstrated here.

The resolution in the \( z_o \) direction can be calculated using formulae describing the propagation of a Gaussian beam. This is not strictly correct, as the slit employed (Fig. 2) means that the beam has a truncated intensity profile rather than a true Gaussian profile. However, the formulae for Gaussian beams can give approximate estimates of the waist size and Rayleigh (waist) length. The half width of the beam waist, \( w_o \), is given by

\[
w_o = \frac{\lambda}{n \pi \theta}.
\]
(for $\theta$ in radians) and so the FWHM of the system described here is estimated to be 4.3 $\mu$m. The confocal parameter, $b$, is twice the Rayleigh range, $z_r$, and is given by

$$b = 2z_r = \frac{2m w_0^2}{\lambda},$$

which yields 81 $\mu$m. Decreasing $\phi_e$ will increase the confocal parameter at the expense of increasing the thickness of the illumination sheet (i.e. a decrease in resolution in the $z_o$ direction shown in Fig. 3).

3. Results

An example of a fluorescence image obtained with oblique illumination of a pollen grain (Carolina, Burlington, USA) collected at IP1 is shown in Fig. 5(a). In this figure there is a central vertical stripe where the pollen grain is in-focus; the obliquely illuminated plane in the specimen rapidly becomes defocused away from this region. A second out-of-focus pollen grain can just be made out above and to the right of the central spiky grain. The corresponding image obtained at IP2 of the same pollen grain is shown in Fig. 3(b). Here the full extent of the optically sectioned oblique image plane is in focus and the second pollen grain is fully apparent.

![Images of two fluorescent specimens obtained with the OPM system.](image_url)
Images of a fluorescently labeled specimen of convallaria (Leica Microsystems Ltd, Milton Keynes, UK) were also recorded at IP1 and IP2 with the specimen again remaining in the same position relative to lens L3 for both images (figs. 3 (c) and (d)), again demonstrating the ability to fully image an obliquely illuminated plane in the specimen. It should be noted that the fluorescence collection efficiency at IP2 is lower than that at IP1 due to the lower NA of the image formed at IP2. Therefore the brightness of the images shown in figs. 5(c) and (d) are not directly comparable. This effect was compensated for by using longer CCD exposure time for figs. 5(b) and (d) compared to 5(a) and (c) and is discussed in detail in section 4. As this is a moderately thick specimen, the fluorescence becomes slightly weaker with increasing depth (towards the right hand side of Fig. 5(d)) due to both absorption and scattering of the excitation light and due to scattering of the emitted fluorescence. A similar phenomenon would also be observed with SPIM and HILO.

To acquire image stacks for 3D rendering, the specimen was mounted on a motorized micrometer stage scanning in the x₀ direction. Due to the oblique nature of the acquired image, the resulting image volume has the shape of a parallelogram in cross-section, i.e. the imaged volume is a cuboid that has been sheared in one direction. The conventional 3-D image can be recovered by numerically shearing the 3-D dataset in post-processing to compensate for this effect. As a demonstration of this 3-D imaging capability, an OPM image stack of 200 images was acquired of the fluorescent pollen grains with a step size in the x₀ direction of 0.5 µm. This data set was rendered using Volocity (Improvision, Coventry, UK) to produce a 3-D image of the specimen, which is shown in Fig. 6.

Fig. 6. 3D rendering of an image stack of fluorescent pollen grains obtained using the OPM system. The axes shown are those of a conventional microscope system (Fig. 3) and the rendered image corresponds to a volume in the specimen of 190×80×50 µm³.

To measure the resolution obtained with this OPM system, a ~20 µm thick sample of orange fluorescent 40 nm beads (Molecular Probes, Invitrogen Ltd, UK) was imaged, as shown in Fig. 7(a). The beads were mounted in glycerol and, although the individual 40 nm beads were not sufficiently bright to be resolved with the CCD camera employed, small sub-resolution clusters of beads could be clearly resolved over the diffuse fluorescent background from individual beads. This sample therefore allowed the uniformity of the excitation beam along the oblique plane to be observed and also provided bright sub-resolution points to provide an estimate of the resolution achieved. Line profiles across 5 separate bead clusters were used to calculate the FWHM, which was found to be 0.82±0.17 µm in the x₀ direction, in reasonable agreement with the theoretical value of 0.67 µm calculated above. An example line profile through a bead cluster is shown in Fig. 7(b).

As with SPIM, the axial response of the OPM microscope in the z₀ direction is defined by a combination of the thickness of the illumination sheet and the axial point spread function.
(PSF) of the detection optics [17]. This means that the axial resolution of SPIM or OPM can be narrower than the thickness of the illumination sheet if the axial PSF of the collection optics is comparable to or smaller than the thickness of the illumination sheet. A direct measurement of the axial thickness of the illumination sheet alone can be derived from the ‘step-response’ of the system to the edge of a semi-infinite fluorescent ‘sea’. This was determined here by recording the 10-90% step response of the OPM system to the interface between the coverslip and the sample of fluorescent beads shown in Fig. 7(a). A line profile was taken from a single row of pixels for five separate images, yielding a 10-90% width of 7.7±0.7 µm in the $x_o$ direction. This corresponds to a 10-90% width of 4.4±0.4 µm in the $z_o$ direction, which is in agreement with the estimate for the illumination sheet thickness obtained above of 4.3 µm.

![Image](image.png)

Fig. 7. (a) OPM image of the sample of 40 nm fluorescent beads, scale bar 20 µm. Two bead clusters at opposite sides of the sample are indicated by white arrows. The brightness and contrast of this image has been enhanced to enable the fluorescent bead clusters to be seen more clearly. (b) example line profile through a bead in the $x_o$ direction and (c) step-response curve obtained from the interface between the coverslip and the bead solution in the sample shown in (a).

### 4. Discussion

The results presented here show that OPM can acquire an optically sectioned image without the use of moving parts or image processing, and a 3D image stack can be obtained by translating the specimen in a single direction. Therefore, provided the sample can emit sufficient fluorescence, 3D datasets could be acquired in around 1 second if a high speed CCD camera acquisition (e.g. at 100’s f.p.s.) is synchronized to the movement of the specimen.

Although OPM effectively requires three microscopes in series, the system is stable and robust, and no day-to-day realignment is necessary. The crucial part of the initial alignment procedure is to correctly overlap the fields of view of lenses L6 and L7. Future improvements in the optomechanical design will allow this to be performed easily and rapidly.
provided that achromatic lenses are used throughout, OPM should be compatible with multicolour fluorescence excitation and detection.

OPM encounters exactly the same problems for imaging in the presence of scattering as SPIM. For the excitation sheet, scattering will cause the width of the excitation sheet to broaden, resulting in a decrease in resolution in the direction perpendicular to the light sheet. For the fluorescence collection, scattering will also degrade the quality of the final image, and this depends on how much scattering is present. As a result, OPM is not directly suitable for imaging in highly scattering turbid media, although these problems have been alleviated for SPIM of scattering specimens through the acquisition of multiple 3D image stacks at different observation angles [7].

Table 1: Calculated values for the resolutions achievable using three commercially available microscope objectives with different immersion media. Lens designations are shown in Fig. 2, and angles in Fig. 4. Values of $\phi_a$ were chosen to give a useful illumination sheet waist length at the specimen. $\delta x_a$, $\delta y_a$ are the calculated FWHM of the point spread function in the oblique plane and $\delta z_o$ is the thickness of the illumination sheet. Excitation and emission wavelengths were assumed to be 532 nm and 610 nm respectively, in line with the wavelengths used elsewhere in this paper. $N_{Apotential}$ is the potential NA that could be achieved by OPM using the lens specified for L3 and assuming an ideal lens for L7.

<table>
<thead>
<tr>
<th>Immersion medium</th>
<th>Air</th>
<th>Oil</th>
<th>Water</th>
<th>Current system</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>1</td>
<td>1.515</td>
<td>1.33</td>
<td>1.515</td>
</tr>
<tr>
<td>L3 (Magnification/NA)</td>
<td>40×/0.95</td>
<td>60×/1.4</td>
<td>60×/1.2</td>
<td>60×/1.35</td>
</tr>
<tr>
<td>$\theta$ (°)</td>
<td>72</td>
<td>68</td>
<td>64</td>
<td>63</td>
</tr>
<tr>
<td>$\phi_a$ (°)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>$\phi_{em}$ (°), eq. (1)</td>
<td>49</td>
<td>40</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>$N_{Apotential}$ (not limited by L7)</td>
<td>0.75</td>
<td>0.98</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td>Potential relative collection efficiency ($N_{Apotential}/N_{AL3}^2$)</td>
<td>0.62</td>
<td>0.49</td>
<td>0.38</td>
<td>0.37</td>
</tr>
</tbody>
</table>

| L6 | 40×/0.95 | 40×/0.95 | 40×/0.95 | 40×/0.85 |
| L7 | 50×/0.5 | 50×/0.5 | 50×/0.5 | 10×/0.3 |
| $f_{L4}/f_{L5}$ | 1 | 1 | 0.89 | 1 |
| $N_{Aactual}$ (limited by L7) | 0.5 | 0.76 | 0.67 | 0.45 |
| Actual relative collection efficiency ($N_{Aactual}^2/N_{AL3}^2$) | 0.28 | 0.29 | 0.31 | 0.11 |
| $\delta x_a$, $\delta y_a$ (µm), eq. (2) | 0.61 | 0.40 | 0.46 | 0.67 |
| $\delta z_o$ (µm), 2×eq. (3) | 3.9 | 2.6 | 2.9 | 4.3 |
| Waist length (µm), eq. (4) | 44 | 29 | 33 | 81 |

The resolution could be improved beyond the values demonstrated in this paper if higher numerical aperture microscope objectives are used and such lenses are commercially available. As an illustration, three alternative configurations are presented in table 1, which demonstrate the improvement in performance that could be achieved over the prototype system presented here. Lens L7 is crucial to the operation of the OPM system since it must
have a reasonably long working distance in order to allow it to be placed at an angle to L6, and it should also have a high numerical aperture to maximize the collection efficiency and achieved lateral resolution. Because there is scope to improve lens L7, the top half of table 1 presents calculations showing the maximum potential performance of OPM that could be achieved with commercially available lenses for L3. The bottom half then shows the actual performance that could be achieved with a commercially available 50×0.5 lens for L7.

It should be apparent that the numerical aperture of the OPM system is reduced compared to the NA of objective lens L3. This reduces the lateral resolution and means that longer acquisition times are required to record images at plane IP2 compared to IP1. This penalty, which is represented in table 1 as ‘Relative collection efficiency’, is the trade-off that must be made in order to implement OPM. With oil immersion and commercially available lenses for L3, L6 and L7, OPM could provide an NA of 0.76 (see column 3 of Table 1) and new long working distance objective lens designs for L7 could increase this to 0.98. Assuming high quality anti-reflection coated optics are employed, such an OPM system could collect up to ~50% of the fluorescence signal collected with lens L3 alone.

5. Conclusion

This paper has presented OPM as a new technique for rapid optically sectioned imaging that is similar to SPIM but offers some advantages, including the ability to image specimens prepared using conventional glass slides and cover slips and straightforward implementation on a conventional microscope system. Compared to SPIM, these advantages come at the cost of reducing the effective numerical aperture used to collect light from the specimen but the achievable resolutions are more than sufficient for most applications. OPM can be combined with other conventional microscopy techniques including bright field and fluorescence microscopy. Here 2D and 3D OPM has been demonstrated with standard fluorescent samples and the resolution of this prototype system has been determined. Using commercially available optics it will be possible to significantly enhance the performance of this technique and the potential improvements have been calculated. The technique of OPM has the potential to achieve a NA of 1 with an oil immersion objective and index matched specimen. For a particular choice of illumination sheet thickness ($\phi_{ex} = 5^\circ$), this would provide a resolution in the plane of the illumination sheet of 0.3 µm and a resolution perpendicular to the illumination sheet of 2.6 µm.

Like SPIM, OPM requires no transverse scanning or image calculations to obtain an optically sectioned image, making it useful for high speed imaging, and it causes minimal photobleaching and phototoxicity since only the plane to be imaged in the specimen is illuminated at any one time. Therefore, OPM may be attractive for time-lapse monitoring of biological samples, e.g. the early development of embryos. Its potential for high speed imaging and its ability to image through coverslips and substrates could be exploited to provide high-speed optically sectioned images of processes occurring within microfluidic devices or high throughput imaging of multiwell plate or biochip sample arrays.

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