Linear versus Non Linear Super Resolved Microscopy

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Classically, microscopes have a fundamental resolution limit due to diffraction. Super resolution techniques may overcome this limit by using a priori information about the input object. In this chapter we discuss the usage of time multiplexing based super resolution technique while comparing between two options of realization one through linear optics and one through non linear one. The linear optics technique is based upon injecting randomly space and time-varied gold nano-particles close to the inspected object. This flow of particles is used as the encoding pattern and since the size of the particles is smaller than the resolution limit of the microscope, proper time multiplexing algorithm allows obtaining a decoding i.e. generation of synthetic aperture with higher NA leading to the recovery of the spatial high resolution features. In the non linear optics technique similar time multiplexing super resolved method is applied. This time the randomly space and time varying encoding structure having high resolution features is obtained due to various nonlinear fluorescence emission effects. The chapter overview various non linear relevant effects and examines the minimal power that is required from the excitation light in order to obtain super resolution. The results show that it is almost impossible to get the nonlinear fluorescence effect without getting being limited by the fluorescence quenching, bleaching and saturation phenomena.

Keywords Super Resolution; nano-particles, fluorescence; photo damage; photo biostimulation; saturation.

1. Introduction

1.1 Microscopy Overview

In 1873, Ernst Abbe discovered that a lens-based optical system cannot distinguish between details that are closer than half of the wavelength of the excitation light divided by the numerical aperture (NA) [1]. This phenomenon is a result of the diffraction of light and depends on the wavelength and the finite size of the objective lenses of a system. Throughout the years, there have been many attempts to bypass the λ/(2NA) limit by developing improving methods all aiming to narrow the PSF of the system and by that obtain high resolution imaging for variety of applications as in biology, chemistry, material science and engineering [2, 3]. Among the most popular techniques is the confocal microscope [4], 4pi microscopy [5], saturation emission depletion microscopy (STED) [6], STORM or PALM [7, 8] and Saturated structured illumination [9] which his results will be discussed later in 5.1.1 section. The various approaches are capable of dealing with sub wavelength features starting from few tens of nm and larger [3, 10]. The improved resolution is most important for biological studies. As many of the biomedical research activities are now focused on the gene and protein level and the interaction in between them, an optical resolution of 10-100nm is essential. Although electron microscopy can provide such high resolution [11], its applicability to most studies is far more difficult than optical microscopy. As an example, in previous studies the telomere dynamics was followed in living cells at a very broad time range of 10^2-10^4 sec [12]. In these studies, both confocal microscopy and wide-field microscopy with convolution were used, but it reached the spatial resolution limit.

1.2 Super-Resolution Overview

Super-resolved techniques are various approaches which are also capable of overcoming this limitation. These techniques convert the spatial degrees of freedom into other domains (encoding), transmit them through the optical system and then reconstruct the image (decoding), based on the classical works of Toraldo Di Francia and Lukosz which describe a system by the numbers of degrees of freedom it can transmit [13-19]. As most of the microscopy methods are aiming to improve the characteristic of the system and by that decrease the width of the PSF, the Super-Resolution techniques in which we presents in this chapter are aimed not to change the system PSF but rather to transmit more information through the same limited system. Due to the fact that the information was previously encoded, and following that choosing the proper decoding implementation, information that beyond the system capability can now be observed. The domains that may be used in order to multiplex the spatial information are the time [16, 20-23], polarization [24, 25], wavelength [26, 27], field of view or spatial dimensions [17, 28-30], code [31-33] and gray level [34].

Time-multiplexing is one of the most applicable techniques for super-resolution. The basic principle demonstrated in Fig. 1 and included the use of two moving gratings [16,20-23] where the first (Fig. 1, red dashed line at plane X0)
encodes the spatial information and is projected or attached to the object [35] and the second (Fig. 1, red dashed line at plane $X_2$), which is responsible for the decoding of the information which was observe through the optically limited system (realize as 4-f system [36] with finite aperture in Fig. 1), is placed near the detector and may be realized digitally by capturing a set of images and multiplying each image in the set by the proper distribution [37]. The two gratings must be different from one image in the sequence to the other by movement in time or space. This relative movement allows the proper decoding of the encoded information.

Later on, instead of using a periodic pattern, a random distribution pattern was used to improve the resolution. The disadvantage of using a periodic pattern is the need to realize and to know the period of the projected sample. In addition the fact that the pattern is periodic reduces the tolerance of the method to system's aberrations. The uses of random a priori known pattern encoded the high spatial frequencies existing in the object. Executing time-multiplexing, one may decode the information and construct the high resolving image of the object. The random distribution can be speckles pattern [38z41], rain droplets [42] or size- varied particles [43].

2. Theory

2.1 Time-Multiplexing Super-Resolution Theory

The operation principle involves the illumination of the sample with a set of high resolution patterns, each one with a slight lateral displacement. The same pattern, corrected with the system magnification, serves to decode each frame. The final reconstructed image is obtained by averaging the images obtained thusly. For simplicity, without loss of generality, we will assume a one dimensional process and a magnification of one.

If we denote by $s(x)$ the high resolution distribution of the object which contains details large than sub-wavelength as well as sub-wavelength features, by $p(x)$ the blurring PSF of our system and by $f(x,t)$ the time-varying random and sparse pattern ($x$ and $t$ are the spatial and the temporal coordinates, respectively), each diffraction limited image in the sequence that is captured by the camera equals to:

$$
\int_s s(x') f(x',t) p(x-x') dx'
$$

(1)

The high resolution decoding pattern is digitally extracted following the numerical procedure explained later on. The reconstruction $r(x)$ is:

$$
\hat{r}(x) = \int_s \int_s s(x') f(x',t) p(x-x') dx' \hat{f}(x,t) dt
$$

(2)

where $\hat{f}(x,t)$ is the digitally estimated decoding pattern. Due to the random distribution of the encoding/decoding pattern we assume:

$$
\int f(x',t) \hat{f}(x,t) dt = \delta(x'-x) + \kappa
$$

(3)

where $\kappa$ is a constant. By changing the order in Eq. (2) and using Eq. (3) we obtain:
The meaning of the result we got in Eq. (4) is that the reconstruction equals to the high resolution object \( s(x) \) multiplied by a constant \( p(0) \) and summed with its diffraction limited version. Thus, by subtracting from \( r(x) \) the diffraction limited version allows us to obtain sub-wavelength features reconstruction that included at the original object \( s(x) \).

The above describes a regular super resolved imaging which contains small than sub lambda features as well as large than sub lambda elements, but in this case the projected pattern must also contains sub lambda features and it must be closer than one wavelength to the object plane.

Loosely speaking, by placing the random high resolution pattern attached to the object allows us to shift the sub-wavelength information carried by the evanescent waves into the non-diffracted range. Thus, the constantly changing of the random distribution allows separation between the sub-wavelength information that were folded into the non-diffracted range and the originally diffraction limited content. Multiplying each image in the stored sequence by the decoding pattern that is similar to the encoding one, a proper resolving of the folded sub-wavelength features is accomplished.

2.2 Sub-Wavelength Encoding Masks

2.2.1 Non-linear Fluorescence

In order to extract the sub lambda information the projected pattern must also contain sub lambda features. One possibility to get such information is to use nonlinear effect. When nonlinearities are present in the process, higher order terms appear in the Fourier transform. The positions of the resulting peaks of the emitted pattern now lie beyond the limiting spatial frequency given by the optical transfer function (OTF) of the imaging system. Although it permits only the detection of rather low spatial frequencies of the emission pattern, the attachment of the Fourier-transformed object to these high frequency peaks allows the detection of object’s spatial frequencies which are beyond the limits obtained by linear pattern excitation. That is, in the nonlinear case higher-spatial-frequency information is shifted into the range of spatial frequencies that is detectable by the system, to a degree determined by the order of the respective nonlinear term. In theory, this circumstance permits the detection of object’s information at arbitrarily high spatial frequencies and therefore with arbitrary high resolution, although in practice the achievable resolution will be limited by the signal-to-noise ratio (SNR) of the raw data.

In order to get non-linear fluorescence phenomena, high illumination intensity is needed. Applying such a single line (or narrowband) light source is not simple. Moreover, such high power might lead to effects that will change the illuminated fluorescent marker, not to mention affecting the sample itself. However, experience shows that such effects occur even at much lower intensities [44, 45].

2.2.2 Gold Nano-Particles Varied in Time and Space

This research expands the approach that was previously shown in [43] into an optical microscopy. In order to observe sub-wavelength information we used SPI-Mark™ un conjugated gold particles with different diameter (200nm or 80nm) and locate them closer than one wavelength to the sample, for encoding the sub-wavelength information which carried by the evanescent waves.

We place those particles in water on top of the sub-wavelength features in order to have time-varied encoding mask. This distribution has the particularity that the particles are sparse, allowing us to extract the high resolution decoding pattern from the diffraction limited images. Since the pattern of each particle is blurred due to the fact that the particle sizes is smaller than half of the wavelength and the blurring spots are spatially separated due to the sparse distribution, their position may be digitally extracted. This fact means that we are extracting the decoding pattern without any a priori assumption regarding the knowledge of the encoding one.

3. Simulation

In order to prove the concept of Sub-wavelength Super Resolution a simulation of the system described at section 2.1 was designed. The virtual microscope was demonstrated with a PSF that uses numerical aperture \( NA=1 \) and optical wavelength of \( \lambda=532nm \). In fig. 2(a) one can see the cross section of the simulated PSF. Figure 2(b) presents the cross section of the high resolution encoding mask \( f(x) \) which relate in this case to a nonlinear emission of a linear speckle.
pattern [38] that was diffused from a Gaussian beam. One can see that some lobes of the speckles are smaller than half of the simulated wavelength ($\frac{\lambda}{2} \approx 250\text{nm}$) which means that higher spatial frequencies can go through the system.

Fig. 2  Simulation parameters. a) Cross Section PSF with $NA=1$, $\lambda=532\text{nm}$. b) Cross section of non linear speckle pattern contains sub-wavelength features.

Figures 3-4 shows the simulation results of the virtual microscope. High resolution image of the target can be seen at Fig. 3. The size of the resolution target is $20\mu\text{m} \times 20\mu\text{m}$ and it contains features bigger than wavelength as well as sub-wavelength features.

Fig. 3  High resolution target.

Figure 4(a) shows the results of “observing” the high resolution target through the virtual microscope using the presented PSF. Such observation is corresponding to the following relation: $I_{\text{out}} = I_{\text{in}} \otimes \text{PSF}$. In Fig. 4(b) the super resolved image after applying the proposed method is presented. One can easily distinguish between sub-wavelength features which cannot be observed through a conventional system. Note that even though this simulation was using a random speckle pattern as the encoding & decoding masks, it shows that using any random mask which contains sub-wavelength features and fulfil Eq. (3), can get us the same significant results.

Fig. 4  Simulation Results of the proposed method. a) Observing the target through the virtual microscope. b) Super resolved image.
4. Material & Methods

4.1 Fluorescence Measurements

The presented work combines the proposed configuration suited to an inverted as well as upright microscope with an obliquely illumination of the inspected biological solutions and cells with blue and green laser at wavelength range of 405-532nm. In the experiment we have used Olympus IX81 and BX51 and objectives of UPlanSApo 10x/0.40 and 60x/1.0. The biological samples were illuminated with blue and green lasers. Image was captured with Pixelink PL-A741-E camera (PixeLINK, Ottawa, ON) having pixel size of 6.7µm X 6.7µm which was connected to the microscope. The output of the Pixelink camera was connected to a computer which was capturing video frames using the manufacturer software. The captured video was analyzed by MATLAB (The MathWorks, Natick, MA) software.

4.1.1 Excitation

A 5W (all lines) argon laser (Spectra Physics and Coherent) and green laser at wavelength of 532nm have used as the source of excitation. The excitation wavelength in most of these investigations was 488nm and 532nm, with intensity at the interrogation point of hundreds of milliwatts.

4.1.2 Emission

In most of the investigations the fluorescent emission was measured either through a long-wavelength cut-off filter (λ>500nm for the blue laser and λ>580nm for the green laser) or a broadband interference filter (500–560nm, 580–630nm) to collect most of the fluorescence emission. All filters are from Chroma (Chroma Technology, Brattleboro, VT).

4.2 Elements Fabrication

For demonstrating our Super Resolved method a target which contains features bigger than wavelength as well as features which are sub-wavelength was fabricated on a Si Dye using focused ion beam (FIB) system. The SEM image of the resulted target is presented in Fig. 5 where the whole size of the manufactured area and the elements S4-S5-S6 are magnified to specify the dimensions.

4.3 Apparatuses Configurations

We used a standard Olympus BX51 upright microscope with a 60X water immersion Olympus microscope objective with a NA equal to 1. Figure 6 shows a picture of the experimental setup. As illumination light we use broad band white light incoming from the halogen lamp of the own microscope illumination system. However and in order to adjust the experimental setup, we place a broad band interference filter (Edmund Optics 950nm with 50nm of FWHM bandwidth).
5. Experimental Results

5.1 Non Linear Phenomenon in Fluorescence

5.1.1 Photosaturation

Photosaturation is the effect in which, beyond certain excitation intensity, no increase in the fluorescence intensity is observed, with increase in the excitation intensity. This phenomenon was already described as a concept for resolution improvement [46]. It was also part of the work was done by Mats G. L. Gustafsson [9] which uses this non linearity of fluorescent layer in order to extract subzwavelength features by illuminating the sample with a periodic pattern. While using structured-illumination microscopy, as Gustafsson described, the number of photons that each molecule can emit before being destroyed is smaller under saturating conditions compared with normal illumination intensities. At the same time, the number of detected photons required for a given SNR is greatly increased compared with a single conventional image. Unfortunately Mats G. L. Gustafsson did not measure normal target, but its target was a fluorescent element. In order to illuminate the target with fluorescence light much higher excitation intensity are needed.

We performed experiments using green laser (\(\lambda=532nm\)) with maximal output power of 400mW and different fluorescence dyes (free Acridine Orange and carboxy SNARF-1 in pH=6) in different concentrations. In all the results non linearity was not reached as can be seen by the solid lines curve of Fig. 7. Although we did not get the non-linear effect we start getting photobleaching. The fluorescence intensity decrease in 35±8% during 3 minutes of continues exposure.

![Experimental layout. a) Olympus BX51 Microscope station, b) 60X water immersion lens with NA=1, and c) water droplet containing gold nanoparticles flowing in proximity to the inspected sample.](image)

![Fluorescence intensity of Acridine Orange (solid thick line) and carboxy SNARF-1 (solid thin line) and U937 cells stained with RH123 (dashed line) for different laser’s intensities.](image)

5.1.2 Photosaturation in the Parallel Direction

When fluorescent markers in biological sample, like cells or beads, are excited by polarized light, molecules whose dipole moment of absorption is parallel to the direction of the exciting field are predominantly excited while those in
other directions absorb less. When the photosaturation process takes place, at the first stage, it leads to an absorption saturation of the parallel dipoles while those at an angle to the exciting field continue to increase their emission when increasing excitation intensity. Apparently, this causes a decrease in the measured polarization. This is a possible explanation of the findings of Keene and Hodgson [47] and Pinkel et al. [48], which showed that the fluorescence polarization of fluorescent beads decreases with increasing excitation energy.

We performed this kind of measurements while using free Acridine Orange and carboxy SNARF-1 in pH6 and measuring the fluorescence intensity using two polarizer that were positioned in parallel to each other, along the optical path of excitation as well as emission. The results for Acridine Orange are presented in Table 1.

<table>
<thead>
<tr>
<th>Laser Power [m Watt]</th>
<th>Fluorescence intensity [arbitrary units]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>963±15</td>
</tr>
<tr>
<td>22</td>
<td>1166±150</td>
</tr>
<tr>
<td>150</td>
<td>31454±220</td>
</tr>
<tr>
<td>190</td>
<td>41685±113</td>
</tr>
<tr>
<td>215</td>
<td>56930±200</td>
</tr>
<tr>
<td>300</td>
<td>72176±205</td>
</tr>
<tr>
<td>350</td>
<td>87421±180</td>
</tr>
</tbody>
</table>

As it can be seen the linear dependence of the fluorescence intensity still exists even at 350mW excitation power and while measuring only the parallel direction of the electromagnetic wave. Similar results obtained for carboxy SNARF-1.

5.1.3 Photobleaching

Photobleaching effects were examined on tens of human U937 cells that were stained with RH123 dye. The results can be seen at the dashed line in Fig. 7 where this results are similar to the fluorescence dyes and beads experiments that are described in Ref. [49]. While measuring from the high intensity 400mW down to few milli-Watts we got the results that are presented in Fig. 7. But when measuring from the opposite direction, meaning from the low intensity level (few milli-Watts) up to the higher intensity (400 mWatt) we got, during 45 seconds of exposure that the fluorescence intensity has decreased by 42±12%. These results can be seen in Fig. 8 which presents the images of U937 cells before and after the 400mW exposure. The images show us that the RH123 dye decrease from the beginning of the measurement (t=0 Fig. 8 middle images) during 45 seconds (Fig. 8 right images) by more than 40%. The meaning is that in normal leaving cells that were stained by RH123 the dye exists in the entire cell, but 400mW illumination bleach the fluoresce dye.

Control measurement done on fluorescent beads (6.5mm diameter, Polyscience, Inc., Warrington, PA, USA) shows that there is no trace of photobleaching in the excitation power ranges of 50 to 400mW. The meaning of the above results is that even at 400mW continues wave laser, nonlinear effects are still not shown but this high power density affects biological samples.

![Fig. 8](image_url) U937 cells as seen at bright field microscopy (left images of both panels) stained with 25µM Rh123 immediately after the first exposure of 400mW (middle images of both panels) and after 45 seconds (right images of both panels).
5.2 Super Resolution of Sub-Wavelength Features Using Gold Nano-Particles

In the experiments we used 30µl SPI-ZMark™ unconjugated gold particles with diameter of 200nm. A set of 400 images were captured in the whole sequence. The camera we used was Pixelink PL-A741-E (PixeLINK, Ottawa, ON) with 6.7µm square pixels, in a frame rate of approximately 130 frames per second. In each image the random distribution of the particles varied due to their Brownian flow in the medium. The number and the positions of the particles in each captured frame are unknown and they were estimated numerically from the set of diffraction limited images. The average number of particles for each frame was approximately 240 which mean that we had 1.2 particles/µm$^2$. Figure 9(a) presents the diffraction limited image of the particles distribution without the resolution test target while Fig. 9(b) shows one diffraction limited image of our target out of the sequence captured by the camera (with the flow of the particles).

![Fig. 9](image1.png)

**Fig. 9** a) Diffraction limited image of the nanoparticles distribution, and b) one diffraction limited image of the resolution target (with the particles) captured by the camera.

Figure 10(a) presents the diffraction limited image of the target which averaged the entire set of captured images. This image is important as part of the numerical process for extracting the decoding pattern and corresponds with the conventional resolution image provided by the microscope lens. According to the theoretical prediction, the resolution limit $R$ provided by the system is 475nm incoming from the relation: $R=\lambda/(2NA)$. As one may see, the last element that can be resolved is marked with a white rectangle and corresponds to bars having a width of 350nm and separated by a gap of 250nm. Finally, Fig. 10(b) depicts the super-resolved reconstruction when performing the proposed approach. One may see that sub-wavelength features corresponding with the elements labeled as S6-S5-S4 which cannot be seen in Fig. 10(a) are now become distinguishable in Fig. 10(b).

![Fig. 10](image2.png)

**Fig. 10** a) Diffraction limited image of the resolution test target obtained when averaging the entire set of captured images, and b) super resolved reconstruction containing sub-wavelength details.

This situation is clearer in Fig. 11 where a comparison of the plot along the horizontal lines of elements S4-S5-S6 in both, the conventional and the super resolved image is presented. The noisy images obtained in the reconstruction of Fig. 10(b) depend on the number of frames taken in the averaging process. The more frames we take, the more uniform and more averaged the reconstruction becomes. Since in this experiment the number of frames was not very large some artifacts related to the particles still remained. Also, some of the nanoparticles were glued to the resolution test target (see Fig. 10(a) and (b)). This is the reason why those nanoscale particles are not avoided in the final reconstructions.
6. Conclusion & Discussion

In this chapter we had presented several high resolution microscopy methods for implementation in variety of applications as in biology, chemistry, material science and engineering. Two new methods that breaking the resolution limit by using time multiplexing techniques have been shown.

Achieving such significant results can be done by attaching a high resolution encoding mask which contains sub-wavelength closer than one wavelength to the desired sample and by that excite the evanescent waves and couple them into harmonic ones.

We have presented a technique which by using fluorescence markers or samples may achieve unlimited spatial resolution. In order to get this, nonlinear dependence of the fluorescence emission rate on the illumination intensity is used. In theory as well as in simulations, in the nonlinear case higher-spatial-frequency information is shifted into the low range of spatial frequencies that may be detectable by the imaging system. As proved in the theory and in the simulations, the nonlinear case permits the detection of object’s information at arbitrarily high spatial frequencies and therefore with unlimited resolution. Unfortunately it is not easy to demonstrate nonlinear characteristics while using continuous wave laser measuring standard biological samples. The intensity that is required in order to obtain good fluorescence measurements is quit high as calculated at the experimental results chapter. Even trying to perform the measurement in lower intensity levels generates a competitive reaction to photobleaching which affects the system and in case of living cell may harm their natural activity. Although highly photo-stable fluorescent labels do exist, they are not common for standard biological samples and non-linear fluorescence phenomenon can not be used.

The proof of principle validation of a linear optical nanoscope capable of provide sub-wavelength super resolution allowing nanoscopic imaging has been also presented. The main advantages of the proposed system are related with: first, a conventional transmission optical working principle; second, a variable, adjustable and unlimited resolution gain depending on the critical size of the inspected sample by only selecting the diameter of the nanoparticles; third, no complex hardware are required since the random nanoparticles movement is provided by Brownian flow motion; fourth, no intense illumination of external laser for imaging the sample; and fifth, it is simple and although validation using transparent sample has been presented, more complex studies including biological samples are in process.

The main trade off concerning the proposed method is the selection of the size of the nanoparticles. There is no problem concerning nanoparticles diameters in the range of 50 nm. However, since the proposed nanoscope uses conventional diffraction limited lenses, the image of the nanoparticles becomes as much blurred as the lower the size is. This problem defines a more sparse distribution of nanoparticles as the particles decrease in size in order to allocate the centers. And this fact increases the time to achieve a wide field super resolution image.

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