Signal-to-Noise Ratio for Confocal Microscopy when Using the Intensity-Modulated Multiple-Beam Scanning (IMS) Technique

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Abstract—The Intensity-modulated Multiple-beam Scanning (IMS) technique involves the use of modulated laser beams for specimen illumination, combined with lock-in amplifiers in the detection channels. In previous studies we have shown that this technique efficiently reduces cross-talk between the channels when simultaneously detecting multiple fluorophores. A drawback with the IMS technique is that it tends to produce images with a lower signal-to-noise ratio (SNR) compared with images recorded without using IMS. Typically a reduction of between 30 and 40% is found. In a previous paper, presenting theoretical derivations of the SNR as well as computer simulations, it was found that the SNR depends on the modulation waveform. In this paper a more comprehensive analysis is made, including non-symmetric waveforms and brief pulses. The effect of a finite contrast ratio for the light modulators is also investigated. It is found that, theoretically, the SNR when using the IMS technique can become equal to that for conventional detection. In practice, however, the SNR is limited by the attainable contrast ratio of the light modulators. Experiments performed are in good agreement with the theoretical results, and by optimizing the modulation waveform we have reached a SNR of nearly 90% of the theoretical maximum.

Key words: Signal-to-noise ratio, confocal microscopy, intensity modulation, lock-in detection, dual labelling.

I. INTRODUCTION

Many confocal microscopes allow simultaneous recording of different fluorophores by spectrally separating the fluorescent light into different bands, each of which is recorded by a separate detector. Multiple excitation wavelengths are often used to efficiently excite all fluorophores. A serious drawback with this technique is that it often produces a strong cross-talk between the recording channels. This cross-talk is caused by the broad and partially overlapping emission spectra of the commonly used fluorophores. To reduce this problem, many confocal microscope systems employ image processing to reduce cross-talk. Although visibly reducing the cross-talk, such methods often introduce artifacts in the images (Carlsson and Mossberg, 1992).

It is possible to eliminate virtually all cross-talk by using the Intensity-modulated Multiple-beam Scanning (IMS) technique (Aslund and Carlsson, 1993; Carlsson et al., 1994). Figure 1 shows a schematic diagram of the IMS set-up. Intensity-modulated laser beams are used for illumination, and lock-in amplifiers (LIAs) are connected to the detectors. The IMS technique makes it possible to use fluorophore combinations that are biologically interesting, but which have hitherto been avoided due to excessive cross-talk.

When using the IMS technique we soon noticed that the images looked noisier than the corresponding ones recorded without using IMS. Investigations showed that, for identical light levels, the signal-to-noise ratio (SNR) for the IMS technique was usually 30-40% lower. This is unfortunate since the noise level is often quite high in confocal fluorescence microscopy and one does not want to increase it further. Experiments showed, however, that the waveform of the light modulation affects the SNR, and therefore a theoretical study with both sine- and square-waves was made (Carlsson et al., 1994). This study showed that square-wave modulation of both the laser light and the local oscillator signal would produce a SNR equal to that obtained for non-IMS recording. This is only attainable if the light is modulated to 100%, which is impossible when using electro-optical modulators (EOMs); in practice one often has to work with modulations in the range 70-95% for frequencies in the MHz region. Furthermore, square-wave modulation of the local oscillator has the disadvantage that it increases the risk of picking up interfering signals from computers and other electronic equipment. For that reason the LIAs in our laboratory set-up (Palo Alto Research, PAR100) use sine-waves.

The present study was carried out to investigate the SNR when using more general waveforms in combination with a light modulation of less than 100%. This called for an extension of the theory presented in Carlsson et al. (1994) to handle non-symmetric waveforms. The aim was to optimize the SNR under realistic experimental conditions, and to find out how much...
could be gained by using non-sinusoidal waveforms. The theory developed is applicable to arbitrary waveforms, but results are presented only for square-waves. There are two reasons for this. Firstly, square-wave light modulation of arbitrary duty cycle and high contrast ratio is easily produced in the experimental set-up, whereas it is more difficult to produce other waveforms. Secondly, the results obtained with square-waves are close to what is theoretically possible regardless of waveform. Therefore, little can be gained by experimenting with other waveforms.

II. CALCULATION OF SIGNAL-TO-NOISE RATIO

For simplicity, it is assumed that the recorded specimen has a perfectly uniform fluorophore distribution, i.e. no structure. The intensity of the illuminating laser beam is assumed to vary according to an arbitrary periodic waveform with a fundamental frequency of $f_0$. The output signal from the detector, $s(t)$, will then vary according to the same waveform. For simplicity, the average value of the signal is set to unity. Figure 2 shows a schematic diagram of a lock-in amplifier. The incoming signal, $s(t)$, is mixed, i.e. multiplied, with the local oscillator waveform, $p(t)$. $p(t)$ also has a frequency of $f_0$ and is phase-locked to the signal modulation, but it is a pure AC wave-form (i.e. time average = 0). After the mixer, the signal is low-pass filtered to remove high-frequency components, yielding an output signal, $S$, of

$$S = \frac{1}{T} \int_0^T s(t)p(t) \, dt$$

(1)

where $T$ is large compared with $1/f_0$. When using non-IMS detection, an identical low-pass filtering effect is provided by the signal integration circuits.

![Fig. 1. Schematic diagram of a two-colour IMS set-up. Electro-optical modulators (EOMs) intensity-modulate the light from two lasers at different frequencies ($f_1$ and $f_2$). The two laser beams simultaneously illuminate the specimen. Two photomultiplier tubes (PMTs) detect green and red parts of the spectrum, respectively. Lock-in amplifiers tuned to the frequencies $f_1$ and $f_2$ are connected to the PMT outputs.](image)

![Fig. 2. Simplified schematic representation of a lock-in amplifier. The input signal, $s(t)$, modulated at frequency $f_0$, is mixed (i.e. multiplied) with the local oscillator waveform, $p(t)$, which has the same frequency. The output from the mixer is low-pass filtered before reaching the output. Since the local oscillator is also used as a master clock for the intensity-modulation of the laser beam, the input signal will be phase-locked to the local oscillator, and the relative phase between the two can be adjusted.](image)
In reality, noise will be superimposed on the signal s(t). In the following analysis, we assume that photon quantum noise is the strongly dominating type of noise. This means that the noise will have a white frequency spectrum up to a limit set by the bandwidth of detectors and amplifiers (in our case 30 MHz). It also means that the root-mean-square (rms) noise value is proportional both to the square root of the signal level, and to the square root of the frequency bandwidth studied. To simplify the calculations we look at only a single noise frequency, f_n, representing what, in reality, would be a small frequency band Δf centred around f_n. We also assume that the rms value of this noise frequency is unity at a signal level of unity. (Since our only purpose is to compare the IMS method with the conventional method, i.e. calculating ratios, the assumptions concerning unity noise rms and unity average signal level are of no consequence.) The noise can then be written as

\[ n(t) = \sqrt{2} \cdot \sqrt{s(t)} \cdot \sin(2\pi f_n t) \] (2)

This noise will also be subject to mixing in the LIA, giving the product n(t)p(t). We must now calculate the noise rms value at frequency f_n, after the mixer, a task that is complicated by the fact that the noise is amplitude modulated at frequency f_0. It will therefore split up into sidebands, creating new components at other frequencies, namely at f_n ± mf_0, where m is an integer number. If the noise spectrum is white, however, there will exist for each sideband that is shifted away from the original noise frequency f_n, an equally large sideband that is shifted to frequency f_n from another noise frequency. (This happens because all noise frequencies will be modulated at frequency f_0.) Therefore, the rms noise value at frequency f_n, n_{rms}, after the mixer will be equal to the rms value of n(t)p(t).

\[ n_{rms} = \sqrt{\frac{1}{T} \int_0^T s(t)p^2(t) \sin^2(2\pi f_n t) \, dt} \] (3)

Combining eqns 1 and 3, and using the fact that the time average of sin^2(2πf_n t) equals 1/2, we get

\[ SNR = \frac{\frac{1}{T} \int_0^T s(t)p(t) \, dt}{\sqrt{\frac{1}{T} \int_0^T s(t)p^2(t) \, dt}} \] (4)

Equation 4 only takes into account a single noise frequency, corresponding in reality to a small frequency band as previously described. The extension to several frequencies is, however, straightforward when dealing with white noise. In the case of m discrete noise frequencies, n_{rms} in eqn 3 is multiplied by \sqrt{m}, and in the case of a continuous frequency band n_{rms} is proportional to \sqrt{\Delta f}, where Δf is the bandwidth of the low-pass filter in . In both cases we simply multiply n_{rms} by a factor that is cancelled when taking the ratio of the SNR for the IMS and the non-IMS methods. Therefore we can use eqn 4 without any further modification in all calculations of SNR.

### III. SIGNAL-TO-NOISE RATIO FOR DIFFERENT WAVEFORMS

Using eqn 4, it is straightforward to calculate the SNR for the IMS technique when using different waveforms of the light modulation and the local oscillator. The SNR for the non-IMS method is always unity, since we have assumed a signal level of unity and a noise rms of unity. Therefore eqn 4 gives the SNR directly for the IMS technique relative to that for the non-IMS technique. It is easily verified that for sine-waves, and square-waves of 50% duty cycle, the results are identical to those presented in Carlsson et al. (1994; Table 1).

For a square-wave signal of duty cycle a (0 < a < 1), and sine-modulated local oscillator, eqn 4 yields

\[ SNR = \frac{\sin(\pi a)}{\pi a} \cdot \cos \phi \sqrt{\frac{1}{2} + \sin(2\pi a)} \cdot \cos 2\phi \] (5)

where \( \phi \) is the phase shift between the signal and local oscillator waveforms. \( \phi \) can be adjusted using the phase control of the LIA (Fig. 2). In an experimental situation the duty cycle a and the phase angle \( \phi \) can be varied to optimize the SNR. Figure 3a shows a 3-D plot of the SNR as a function of a and \( \phi \). It is clear that both a and \( \phi \) must be small to obtain optimum SNR. However, there is a relatively large region of a- and \( \phi \)-values where the SNR varies little, and therefore the choice of these parameters is not critical. Optimum adjustment of \( \phi \) is easily obtained by maximizing the signal level, which is proportional to cos \( \phi \) (the signal being represented by the numerator in eqn 5). The variation of SNR with a for the case \( \phi = 0 \) is shown in Fig. 3b. In the range 0 < a < 0.3 the SNR is almost constant, and therefore very little is gained by using a lower duty cycle than 0.3.

As mentioned in the introduction, a non-sinusoidal local oscillator waveform increases the risk of picking up interfering signals. Nevertheless, many LIAs use square-waves in the local oscillator, and an input is often provided so that the user can connect an arbitrary waveform to be used as local oscillator. It is therefore interesting to study what effects this will have on the SNR, provided interference can be avoided. This has already been done by Carlsson et al., 1994 for the special case of a square-wave of 50% duty cycle. If both signal

<table>
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<tr>
<th>EOM waveform</th>
<th>Local osc. waveform</th>
<th>SNR</th>
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<tbody>
<tr>
<td>Sine</td>
<td>Sine</td>
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<tr>
<td>Square</td>
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</tr>
<tr>
<td>Sine</td>
<td>Square</td>
<td>0.637</td>
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<tr>
<td>Square</td>
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Table 1. Calculated signal-to-noise ratios for the IMS technique for different combinations of EOM and local oscillator waveforms. In all cases involving square-waves a duty cycle of 50% is assumed.
and local oscillator are square-wave modulated, the result is a SNR of unity, i.e. the maximum possible. Using eqn 4, it is easily shown that this is true regardless of the duty cycle of the square-wave, provided that the signal and local oscillator waveforms have identical duty cycles. It is therefore possible to use a duty cycle approaching unity, and this has two advantages. Firstly, it means that lower output power from the laser is needed to produce the same average specimen illumination (in all cases studied we assume that the average light intensity in the specimen is the same). Therefore, a smaller laser is sufficient. Secondly, the maximum light intensity in the specimen is reduced, which reduces the risk of saturation effects in the fluorophore.

So far, we have assumed that the signal is modulated to 100%, i.e. that it drops to zero during some part of the period. This requires that the EOMs are able to completely block the laser light, which is not possible in practice. The contrast ratio of an EOM, $I_{\text{max}}/I_{\text{min}}$, can vary considerably depending on wavelength, beam diameter, modulation frequency, and the amount of distortion tolerated. For the EOMs used in our laboratory (Conoptics 380-2P) the contrast ratio is below 50 at the wavelengths and modulation frequencies used in the IMS experiments.

The effects of a finite contrast ratio can easily be incorporated into the calculations based on eqn 4. In these calculations it is more convenient, however, to use the inverse contrast ratio, $b = I_{\text{min}}/I_{\text{max}}$, as a variable. Assuming $\phi = 0$, we get for square-wave signal and sine-wave local oscillator

$$\text{SNR} = \frac{1 - b}{\pi(a + b - ba) \cdot \sin(\pi a)} \cdot \sqrt{\frac{1}{2} \cdot \frac{1 - b}{4\pi(a + b - ba)} \cdot \sin(2\pi a)}$$

Equation 6

Figure 4 is a graphical illustration of eqn 6, showing SNR as a function of duty cycle, $a$, and inverse contrast ratio, $b$. For increasing values of $b$ two effects are visible: the maximum SNR is progressively reduced, and the maximum occurs at larger values of $a$. Thus, for $b = 1/25$, a typical value in a real situation, the maximum SNR is 0.89, for $a = 0.33$.

Also, when using square-waves for both signal and local oscillator, the SNR will depend on the contrast ratio. In this case eqn 4 gives the following result

$$\text{SNR} = \frac{1 - a - b + ba}{\sqrt{(\frac{1}{a} - 2) (a + b - ba) + (a + b - ba)^2}}$$

Equation 7

An illustration of this is shown in Fig. 5. Compared with Fig. 4, the SNR levels are higher and display a much weaker dependence on the duty-cycle $a$. For $b = 1/25$ the maximum SNR is 0.92, which can be compared with the value of 0.89 obtained for the sine-modulated local oscillator. The difference between sine- and square-waves in the local oscillator is therefore marginal in practice.
IV. EXPERIMENTS

In order to test the validity of the theoretical derivations, experiments were carried out for four different signal waveforms. Thus, sine-waves and three different square-waves ($a=0.1, 0.35, \text{ and } 0.5$) were used to modulate the laser light. To create square-wave modulated laser light with a variable duty cycle, the local oscillator sine-wave was used to trigger a function generator, whose output signal was used, after amplification, to modulate the EOM. In this way the light modulation was phase-locked to the local oscillator as required for lock-in detection. In all experiments the laser wavelength was 488 nm, the modulation frequency 0.48 MHz, and the local oscillator waveform was sinusoidal.

To obtain stable experimental conditions a flat mirror was used as a specimen. Images with a size of $512 \times 512$ pixels were recorded with one scanning mirror stationary, thereby giving 512 repeated recordings of a single scan line. In each scan line only about 10% of the pixels near the centre were used so that problems due to non-uniform light distribution across the field were avoided. Thus, for each 'image' about 25,000 pixels were selected for measurement. For these pixels the mean value and the standard deviation were calculated. The dark level, which was mainly due to electronic bias in amplifiers, was recorded separately and subtracted from the mean intensity value. During the experiments the laser (Coherent Innova 70-2) was running in the light control mode at an output power of approximately 100 mW. Under these conditions intensity variations in the beam were only about 0.5% rms, and therefore negligible compared with the photon quantum noise. To ensure that photon quantum noise was the strongly dominating source of noise, all image recording was made at low light levels, corresponding to about 100 detected photons per pixel. Under these circumstances other types of noise were at least an order of magnitude smaller than the photon noise, and therefore influenced the measurements by less than 1%. Nevertheless, this additional noise was measured and compensated for, based on the assumption that the different noise sources were incoherent.

For each waveform four images were recorded, one using lock-in detection (IMS), one without lock-in detection (non-IMS), and two corresponding dark images (i.e. with the light path blocked). From these images the SNR for the IMS and non-IMS methods was calculated as follows

$$SNR = \frac{I - I_d}{\sqrt{\sigma^2 - \sigma_d^2}}$$  \hspace{1cm} (8)

where $I$ is the mean light intensity and the standard deviation. Subscript $d$ denotes dark image values. The SNR value for the IMS method was then divided by the value for the non-IMS method to provide a normalized value. Each experiment was repeated ten times to assess the repeatability of the measurements. In Table 2 the averages and standard deviations are presented. As expected, when using the IMS technique the SNR is higher for square-waves than for sine-waves. Reducing the duty-cycle of the square-wave below 50% improves the SNR, but at very low duty-cycles the SNR becomes worse. This behaviour is in good agreement with the theoretical results shown in Fig. 4. Assuming a contrast ratio of 25, which is a realistic value, a good fit is obtained between the theoretical results and the measurements, Fig. 6. The real contrast ratio was estimated from an oscilloscope screen displaying the modulated laser beam detected by a photo-diode. This only permitted a rather crude estimate in the range 20–40, which nevertheless agrees well with the value used in Fig. 6. It should be pointed out that due to temperature drift in the bias setting of the EOM, the contrast ratio varied considerably over time unless compensation was made for this. During the experiments the modulation waveform was therefore continuously monitored on an oscilloscope screen and the bias voltage frequently adjusted.

![SNR for square-wave-modulated signal and sine-modulated local oscillator as a function of the duty-cycle $a$ for the square-wave ($b=1/25, \phi=0$). Experimental results are indicated by diamonds.](image)
In addition to the experiments with a reflecting specimen, measurements were also made using a solution of free fluorophore. The purpose of this experiment was to demonstrate that similar results are obtained with both fluorescent and reflecting specimens. A 0.05 mg/ml solution of Texas Red in ethylene glycol was used for this experiment. The conditions concerning laser wavelength, modulation frequency and so on were the same as in the previous experiments, with the exception that a 550 nm long-pass barrier filter was used in front of the detector to suppress reflected laser light. Because of photobleaching the conditions could not be expected to be as stable as with a reflecting specimen, but no reduction in light intensity could be observed even after prolonged scanning periods. The average illumination intensity in the specimen was approximately 0.1 mW in these experiments. Evaluation of the measurements was done in the same way as previously described, but the measurements were repeated only three times instead of ten as for the reflecting specimen. Measurements with square-wave modulated laser beam and duty cycles of 50, 35, and 10% gave SNRs of 0.80 ± 0.01 (average ± SD), 0.86 ± 0.01, and 0.82 ± 0.01 respectively. These numbers are similar to the ones obtained with the reflecting specimen, and the same general trend in the variation of SNR with duty cycle is clearly evident. For sine-modulation the SNR was 0.67 ± 0.01 which is also close to the value obtained for the reflecting specimen.

To test the assumption that the noise has a white frequency distribution, a spectrum analyzer (Tektronix 2712) was connected to the detector output (before the lock-in amplifier in the red channel in Fig. 1). The displayed spectrum was perfectly uniform (deviation <1%) for frequencies up to 1 MHz. Also in the range 0–5 MHz the deviations were small, less than 10%. Since the modulation frequency of the laser light was 0.48 MHz, the noise spectrum could be considered white up to at least the tenth harmonic of this frequency. Therefore, the assumptions underlying eqn 3 seem valid.

### V. DISCUSSION

The results listed in Table 2 verify that a substantial improvement can be made by properly selecting the modulation waveform for the illuminating laser light. Compared with sine-modulation, square-waves with a duty cycle of approximately 35% result in an improvement in SNR by a factor of 1.3. Such an improvement, which corresponds to a 70% increase in the light intensity measured, is clearly visible in the recorded images. It is easy to implement square-wave EOM modulation in the experimental set-up. All that is needed is to insert a Schmitt trigger between the local oscillator output (sine-wave) and the input of the EOM driver amplifiers. On the other hand, the marginal improvement offered by using a non-sinusoidal local oscillator waveform does not seem worthwhile even though the lock-in amplifiers allow for an external local oscillator waveform to be used. In this case, however, the user has to provide for the phase control (cf. Fig. 2) which complicates the modification. Furthermore, a non-sinusoidal local oscillator waveform increases the risk of picking up interfering signals as pointed out earlier.

It was mentioned in the previous section that the maximum contrast ratio of the EOM used is in the range 20–40. This maximum contrast ratio can be utilized with square-waves, but for sine-waves heavy distortion will occur due to the non-linear behaviour of the EOM. In order to produce sine-modulation without any visible distortion the contrast ratio must be reduced to somewhere in the range 5–10, which was done in the measurements described. On the other hand, if a distorted sine-wave is accepted a higher contrast ratio can be used, and SNR values of about 0.75 can be obtained. This value is higher even than the theoretical value of $1/\sqrt{2}$ for sine-wave-modulation and infinitely high contrast ratio. Obviously the distortion of the sine-wave improves the SNR, and this improvement more than compensates for the finite contrast ratio. Therefore, in practice, it is less of a disadvantage to use (slightly distorted) sine-waves than it would appear from Table 2.

Looking at Figs 4 and 5, it is clear that the contrast ratio of the EOMs must be high in order to obtain a high SNR. As described in the previous section, the bias setting of the EOMs is not stable over time, which can result in large variations in contrast ratio. It is therefore important to keep this drift under control. We plan to do this by installing an automatic bias control that monitors the light output from the EOMs and adjusts the bias voltage when necessary.

### REFERENCES


### Table 2. Measured signal-to-noise ratios and standard deviations for the IMS technique (n = 10). In all cases a sine-wave was used as local oscillator waveform. $a$ = duty cycle of the square-wave

<table>
<thead>
<tr>
<th>EOM Waveform</th>
<th>Average SNR</th>
<th>SD</th>
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<tbody>
<tr>
<td>Sine</td>
<td>0.689</td>
<td>0.013</td>
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<tr>
<td>Square, $a = 0.5$</td>
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<td>0.007</td>
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<tr>
<td>Square, $a = 0.35$</td>
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<td>0.011</td>
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<td>Square, $a = 0.10$</td>
<td>0.811</td>
<td>0.021</td>
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