**Image-Scanning Microscopy and Stochastic Optical Fluctuation Imaging: Making it easy and user-friendly**

Dirk Hähnel, Simon Christoph Stein, Anja Huss, Ingo Gregor, Jörg Enderlein  
III. Institute of Physics – Biophysics, Georg-August-University Göttingen  
Friedrich-Hund-Platz 1, D-37077 Göttingen, Germany

Email: [enderlein@physik3.gwdg.de](mailto:enderlein@physik3.gwdg.de), Internet: [www.joerg-enderlein.de](http://www.joerg-enderlein.de)

**ABSTRACT**

Recent years have seen a tremendous increase of new and novel methods of high and Superresolution fluorescence microscopy1, going beyond the Abbe resolution limit2. Among them, our group has developed to powerful methods: Confocal Spinning Disc Image-Scanning Microscopy (CSDISM)3 4, and Superresolution Optical Fluctuation Imaging (SOFI)5 6. However, new microscopy techniques that provide not only enhanced image quality and resolution, but they are also simple enough for finding broad application. Here, we present embedding solutions for both CSDISM and SOFI which enable potential users to implement them in an easy and straightforward way into their existing microscopy systems. In the case of CSDISM, we have integrated the method into the environment of the widely used and popular MicroManager Open Source Imaging platform. This allows any researcher who already has a commercial Confocal Spinning Disk microscope to easily implement the image-scanning option and thus to double the spatial resolution. For SOFI, we have developed a dedicated hardware based on a Freely Programmable Gate Array (FPGA) which converts, in real time, image movies taken by high-speed CCD systems into SOFI cumulant images. Thus, all algorithmic complexities and numerical workload of SOFI calculations are taken care of.

1. **INTRODUCTION**

Super High Resolution (SHR) microscopy is a relatively young scientific area, which is framing a diverse group of scientific disciplines. Tremendous insights were gained over the past decade by biochemists, biophysicists and biologists. Nevertheless the subfield of SHR imaging in the field of bioinformatics is relatively inequitably represented, thus we lack a wide range of open source software tools to provide access of those SHR imaging technics to the non-expert group. The investigation of algorithmic complexity for the SHR microscopy would give inference to the general applicability and practicableness of the underlying scientific method. As a consequence of that approach, our aim is to investigate the algorithmic part of our recently developed SHR microscopy methods, termed SOFI and CSDISM. We will present an integrated software approach for the CSDISM which yields every regular spinning disk microscope into a CSDISM microscope with increased resolution. Regarding SOFI microscopy we will present our ongoing work to massively parallelize the algorithm for the image formation within a FPGA processor design.

* 1. **SOFI**

The Superresolution optical fluctuation imaging (SOFI) method was developed recently and might easily be distinguished from most other Superresolution imaging technics, by its inherent robustness and simplicity. Instead of switching the molecules sequentially or simultaneously, as it is required for PALM/STORM7–10, the SOFI5,6 algorithm derives superresolved information only from stochastic11–15 “On” and “Off” states in the temporal domain. Required is the intensity fluctuation of the emission of a molecule over the acquisition time. The root of that intensity fluctuation can technically be anything, from changes in polarization or the alternating bright or dim fluorescence states. The emitters must fluctuate stochastically and independent, thus are not interacting with their neighbor emitters, hence a labeling density greater than 10 nm is essential. In a sample composed of independently fluctuating emitters at location with time dependent molecular brightness, the resulting fluorescence source distribution can be derived with:



Where represents the time dependent fluctuation furthermoreis the constant molecular brightness. The fluorescence signal at position and time is given by the convolution of the Point Spread Function (PSF) with the fluorescence source distribution:



Hence the observable molecules are in stationary equilibrium whilst acquisition, thus fluctuations can express as zero-mean fluctuations:



The most primitive case is the second order autocorrelation function:



Hence all cross correlation terms  where  vanish, the second order autocorrelation function reduces to a sum of the squared PSF. Weighted with the squared brightness of each emitter and molecular correlation function. Thus the SOFI image is defined by the value of  with time lag. The intensity of the SOFI image is derived by the brightness and degree of correlation of the fluorescence signal. The PSF is represented by a distribution that is the squared original PSF. It follows from that the width of the new PSF can be reduced by  in all dimensions. Going from the second order correlation function to higher order correlation functions is the next logical step to further increase the resolution, the definition of the  order correlation functions is:



This can be written as:



The acquired signal fluctuations have to be multiplied for  time lags to generate. Thereafter one can generate higher order SOFI images with transforming higher order correlation functions into higher order cumulant functions. More precise, the  order correlation function represents the  order cumulant function. Hence the cross term contributions of the lower order correlations are eliminated in the  order cumulant function. Thus only terms containing the  power of the PSF are contributing to the order cumulant function. The higher order cumulant function is the following:



The algorithmic complexity yields exponentially for higher order SOFI images where all information from the signal source is used for the final image formation, due to the fact that the  order cumulant is a  dimensional function of. For subpixel granularity, which is not described here in detail, because it would go beyond the scope, one must consider even further rise of algorithmic complexity. The approach of cross correlation for example, can easily escalate into a few hundred thousand combinations, hardly solvable without dedicated computer cluster.

* 1. **ISM**

The Image Scanning Microscope (ISM) method utilizes a standard confocal microscope, but the point detector is replaced by an image detector, utilizes the similar principle structured illumination microscopy (SIM)16 17. Thus one is able to record the full illuminated region at each scan position, in particular the full PSF for that particular scan step. Hence the ISM image can be expressed mathematically3 18 4:



The scan position of the scanner is marked; the position on the CCD is represented with. Whilst integrating  over  the image can be recovered by the confocal microscope. If the data is shifted in  space by the value of  and afterwards integrate over, the image recovering is obtained as in a wide field microscope. The excitation intensity distribution is multiplied with the shifted PSF , since the center of gravity of the product  is shifted by  , which is exactly half the width of a scan step, one must shift the this center of gravity back to the center of the optical axis by the and afterwards integrate over . Thus the image and the PSF can be expressed mathematically in the following form:





The acquired signal on the CCD at the position, with the responding position  in sample space, is added to the final image at the position  at image space. However, the most nontrivial part of the ISM method is the data acquisition. In general it is possible to functionalize a standard laser scanning microscope (LSM) enhanced with a multi pixel detector device, ether an array of photon counting avalanche diodes or an CCD. It is very crucial to synchronize the scanning position  with the signal acquisition device. Since most LSM use oscillating galvo-scanner, the motion of the scan unit is way ahead of the physically achievable photon acquisition with a CCD detector. Thus a slow piezo scanner would fulfill the requirements; Problematic with this approach is the temporal resolution of the overall imaging procedure, every single scan step last’s at least. This could be expressed mathematically:



In addition to the non-ideal temporal resolution – for an image ofthe temporal resolution would be roughly between three and four minutes. Besides, the mechanical accuracy of the piezo positioning system is insufficient as well for the linear and nonlinear sample scanner movement. We found that a continuous movement of the scanning device was impossible to conduct whilst keeping the synchronization time jitter for of sample space position and the detection device below the acceptable threshold. Hence we decided to move to a faster acquisition technic with less negative side effects in terms of device synchronization. Considering enhancing a confocal spinning disk setup with ISM enables us to utilize a greater sampling area and shorten the acquisition time drastically. Thus, we are able to increase the temporal resolution to investigating biological process, so called live imaging. The confocal spinning disk image scanning microscope consists of two synchronously rotating disks, one disk with several microlenses and their aligned pinholes on the other disk, arranged in Nipkow geometry. The two aligned rotating disks have a fixed frequency which leads to a focal spot frequency of. To illuminate the sample at the correct position of the discs, four short laser pulses are generated in each quadrant. To further increase temporal resolution one can easily subdivide into octants or even sixteenths. Each subinterval is defined from; several delayed illuminations are performed until the sample space is screened completely. Hence one needs to implement two synchronized oscillators, the second with a slightly slower frequency than the other. The crux is to phase couple them at the beginning of the acquisition in their zero crossing moment.

1. **IMPLEMENTATION**

In this section we describe about the implementation of the two SHR microscopy methods into our setups, thus enabling use to run our experiments with ease. The intention was to use a many freely on the market available parts, thus enabling the scientific community to recreate them. For simplicity and openness, we have chosen to enhance the widely distributed Micromanager Software with our two SHR imaging methods.

* 1. **SOFI**

We have investigated many different approaches to find a discrete algorithm which solves the cumulant, not only chasing for velocity but also numerically stable. Analytically the mean for all combinations of time windows will reconstruct the first order SOFI PSF, the variance for the second order SOFI PSF, since the second order SOFI image reconstruction is a simple autocorrelation. Considering a random process as a data generating function, the statistical output of the data set , can be described by its moments or by its cumulants. Comparing equation we can describe the combination of all intensity values, at different  as probability density function. If we write the intensity signal as a vector



and considering the values  as a mixture of underlying signals  coming from different sources, more exact, different emitters and disregard components as noise and the nonlinearity in the intensity distribution of the emitters for different higher order spectra (HOS) , which means that an intensity value from a HOS is dimmer than from the second order case. Going back to the discrete solution one can start to describe the problem as:



Where  denotes to the intensity value and  represents a mixing matrix which solves for the final intensity value. Problem here is to define the mixing matrix, best approximating the real value. We found that the best approach is a Richardson Lucy deconvolution, but in general which underlying distribution the mixing matrix represents can be neglected in this paper, since we are more focusing on the computational aspects. To reconstruct the second order SOFI image, one might think to use a Fast Fourier Transform (FFT) and perform a frequency domain analysis will lead to the least complexity and thus generating a feasible runtime estimation; The downside is that the memory requirements for the image buffer are correlated to the desired frequency accuracy, secondly the length buffer must be fixed. FFT is necessary to receive subpixel accuracy, see our recent fSOFI paper for more details. Therefore we have to find solutions for fast throughput of the acquired data, which we currently investigating. The general flow can be described as the following:



Figure 1: Fast Image Reconstruction Pipeline

First the incoming intensity values must be transformed to Fourier Space and then additional subpixels can be introduced. Finally the discrete deconvolution can be performed to reconstruct the final image. The underlying algorithms for the online FFT and deconvolution technics and the more important buffering procedures will be part of a future publication, thus not outlined here in more detail.

* 1. **ISM**

The CSDISM method inherits critical timing requirements to synchronize the acquisition procedure, thus an external coprocessor for the trigger generation is necessary. For practicability reasons we decided to use a freely available Field Programmable Gateway Array (FPGA) processing unit, deployed on a hardware board equipped with a Peripheral Component Interconnect Express (PCIe) bus interface. The processor is a XILINX Vertex II but in general any processor with a Digital-Input-Output (DIO) cycle time in the range of some tenth of nanoseconds would be sufficient to set the synchronization timings correctly. We have chosen an integrated encapsulated FPGA design to utilize the over three million logic gates for the CSDISM image formation as well. We will now discuss the calibration procedure and the architecture of the different modes in more detail.

* + 1. **Encoder**

The most important part for the CSDISM is the calculation of the velocity and acceleration of the spinning disk which drives the other components, as there are CCD camera exposure triggering and the laser pulses which need to be super accurate to illuminate the sample at the correct disk position only.



As outlined in equation the amount of clock ticks divided by the tick signal from the disk over a fixed time interval  represents the disk velocity. Now the important thing is the acceleration, because we are interested to compute position estimates for the next disk rotation  or at least to correct for eventual shift in time for the actual rotation cycle of the disc .



With equation we are able to calculate the acceleration for each time interval, which then leads to the adjustment of the trigger events to maintain equal spacing between the multiple foci spots until the sample space is fully screened by the disc rotation.

* + 1. **Calibration**

In this step, we have to map the position of the multiple foci on the rotating disk with the position on the CCD detector. To correctly identify the coordinates of the focal spots at each timestamp at the illumination pulse train, we use calibration samples, consisting of a surface area, heavily inked with some labeling dye. Thereafter the measuring procedure starts and after the full sequence is processed, the focal positions appear as bright illumination spots on the stacked images. Eventually one needs to fit a 3D Gaussian model to the data to accurately estimate the center position with subpixel granularity. Those calibration data is applicable until the real measurement won’t be performed with a different set of parameters. To name a few exemplarily: rotational speed of the disks, wavelength of the excitation laser, different type of dye etc. The reason for doing so is comprehensible, say using 647nm calibration data for~380nm excitation patterns, but we encourage to better bring into line every new set of parameters with a proper calibration measurement.

* + 1. **CSDISM – Synchronization Mode**

In this configuration the FPGA coprocessing is deployed on a hardware board without camera link interface. This reduces the overall setup price by 80%, thus roughly doubles the resolution of a standard CSD with less than one thousand dollar investment for additional hardware. The synchronization between the rotating disks, the laser for illuminating the sample and the detecting device is performed on the FPGA. A correction mechanism for compensating clock drift is implemented as well. The Acquired images are transferred via micromanager image pipe directly from the detecting camera into the host computer memory. To reconstruct the SHR image, we implemented a micromanager plugin which performs image formation. The main difference to the fast mode, which is explained in the section below, is that the acquisition and reconstruction are synchronized, but sequential procedures. Hence, the total acquisition time with fastest frame rate is somewhere around  minutes and the live SHR image screen is delayed.

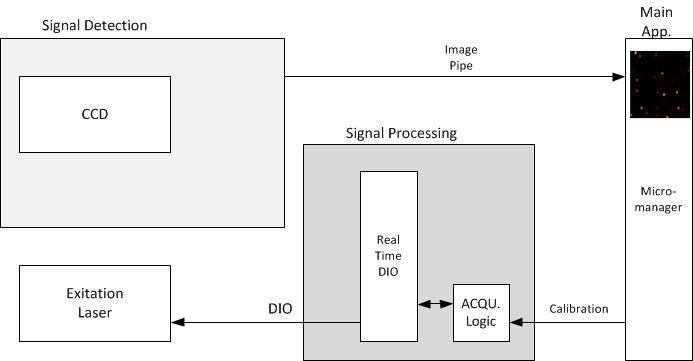


Figure 1: CSDISM System Integration Chart

* + 1. **CSDISM Synchronization and FastCSDISM Image reconstruction**

In this configuration the complete CSDSIM takes place on the external coprocessing unit. Firstly, the photon detection device CCD is connected via camera link interface directly to the hardware board which hosts the FPGA. Since the data transfer is carried out with a maximum transfer rate of  the acquisition time defines the lower limit in terms of acquisition time. Fortunately, the image reconstruction contains only simple mathematics, thus the FPGA can play out its inherent velocity advantage. Thus the recent acquired images are directly processed with the FPGA to parallelize image acquisition and image reconstruction. Finally after one acquisition cycle the ISM image with enhanced resolution is transferred from the hardware board to the host computer. The fantastic development allows for a SHR live screen with minimal delay, put in other words, the SHR image is shown on the experimenters display right away with negligible delay. Secondly, there is practically no limitation with streaming capacity on the host computer, since only the final SHR image is transferred to the host application. In this case we increase the total image size by the factor of two, meaning multiplication of all pixel positions by the factor of two, thus the intensity signal can easily be added at the center of the pixel position of the final image. To enable us to add the signal from every acquired frame to the right position in the final image at every tick of the FPGA processor, we need to think of a smart adder design which can calculate the carry and the sum at a specific position at the same time19.



Let us consider  as the intensity value from the CCD signal at position, with position  in sample space which will be summed at position  in the final SHR ISM image. In our design, we need to consider, that there are intensity values from many positions of the signal space being terms of the summation which adds up to the final intensity value  at the final image. For simplification we simply consider  as the intensity added to the sum of all intensity components. The function generates, if and only if both input bits are true, in other words, if. The term  propagates, if at least one of the inputs bits  or  is true, formal definition is  . The third equation  utilizes a xor logic processor instead, which is faster in practice and used, but for simplicity we use the simple propagation instead. The addition will carry, if ether the addition generates or the next less significant bit carries and the addition propagates. Every intensity value, ether of the SHR ISM image or the value being added to it at the determined position must be expressed as a sequence of bits. If now those two paired bits are added, the logic will generate a carry or propagate a carry. The magic is now, that the logic can predetermine the carry ahead of time and for the actual addition the circuit mustn’t wait for the carry. The carry for say, the  bit of a sequence can be generated with inserting the lower  into the equation:



Where functions as generator andas propagator. The underlying algorithms and results will be part of a future publication, thus we decided to present the most significant parts in this contribution.

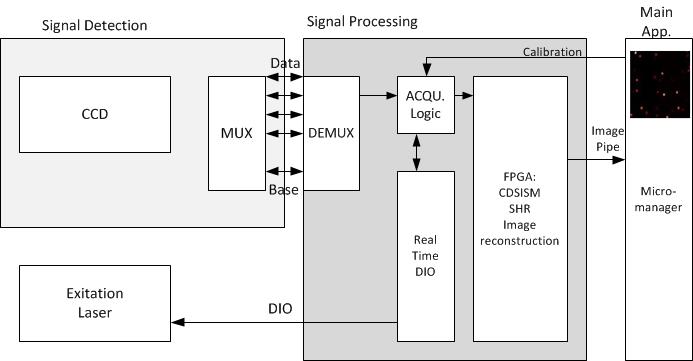


Figure 2: Fast Live CSDISM with Hardware Image Reconstruction

**REFERENCES**

1. Hell, S. W. & Wichmann, J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* **19,** 780–782 (1994).

2. Abbe, E. Contributions to the theory of the microscope and microscopic detection (Traduction from German). *Arch. für Mikroskopische Anat.* **9,** 413–418 (1873).

3. Müller, C. B. & Enderlein, J. Image Scanning Microscopy. *Phys. Rev. Lett.* **104,** 1–4 (2010).

4. Schulz, O., Pieper, C. & Clever, M. Resolution doubling in fluorescence microscopy with confocal spinning-disk image scanning microscopy. *Proc. …* (2013). doi:10.1073/pnas.1315858110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1315858110

5. Dertinger, T., Colyer, R., Iyer, G., Weiss, S. & Enderlein, J. fluctuation imaging ( SOFI ). *PNAS* (2009).

6. Dertinger, T., Colyer, R., Vogel, R., Enderlein, J. & Weiss, S. Achieving increased resolution and more pixels with Superresolution Optical Fluctuation Imaging (SOFI). *Opt. Express* **18,** 18875–18885 (2010).

7. Zhu, L., Zhang, W., Elnatan, D. & Huang, B. Faster STORM using compressed sensing. *Nat. Methods* **9,** (2012).

8. Zhuang, X. Nano-imaging with Storm. *Nat. Photonics* **3,** 365–367 (2009).

9. Geissbuehler, S., Dellagiacoma, C. & Lasser, T. Comparison between SOFI and STORM. *Biomed. Opt. Express* **2,** 408–20 (2011).

10. Rust, M. J., Bates, M. & Zhuang, X. imaging by stochastic optical reconstruction microscopy ( STORM ). 5–7 (2006). doi:10.1038/NMETH929

11. Mendel, J. M. Tutorial on Higher Order Statistics (Spectra) in SIgnal Processing and System Theory: Theoretical Results and Some Applications.

12. Brillinger, D. Asymptotic theory of estimates of kth order spectra.

13. Brillinger, D. An introduction to polyspectra. *Ann. Math. Stat.* (1965). at <http://www.jstor.org/stable/10.2307/2238424>

14. Candes, E. J. & Donoho, D. L. Recovering edges in ill-posed inverse problems: Optimality of curvelet frames. *Ann. Stat.* 784–842 (2002). at <http://www.jstor.org/stable/10.2307/2699979>

15. Geissbuehler, S. *et al.* Mapping molecular statistics with balanced super-resolution optical fluctuation imaging (bSOFI). *Opt. Nanoscopy* **1,** 4 (2012).

16. Gustafsson, M. G. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* **198,** 82–7 (2000).

17. Gustafsson, M. G. L. Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 13081–6 (2005).

18. Sheppard, C. J. R. super resolution in confocal imaging.

19. Zlatanovici, R., Kao, S. & Nikolic, B. Energy-delay optimization of 64-bit carry-lookahead adders with a 240 ps 90 nm CMOS design example. *IEEE J. Solid-State Circuits* **44,** 569–583 (2009).