

# Paradigm shift in laser scanning confocal microscopy:

## Resonant real time live spectral imaging for cell dynamics

Vee-Jay Light, Gene Maverick

### Introduction

Cell underlies the basis of all living organisms that operates both simple and complex arrays of diverse biochemical and molecular processes, including those that govern its own growth, division, development and survival. Understanding these complex cellular behavior in terms of its morphology, topography, physiology and the function is a complicated task and perhaps the most important when such cellular processes break down suddenly, causing disease and ultimately death of a organism. The use of cell imaging fluorescence microscope with high spectral resolution has enabled live cell imaging using confocal laser scanning microscopes. The temporal and spectral resolution capabilities of confocal systems are especially useful for detecting spectral changes of a fluorescent dyes that reveal the complex cellular and molecular dynamic processes.

Nikon has designed a new confocal system A1 to cater the upcoming needs of live cell imaging and molecular interaction analysis. As the molecular biological processes are happening *in-vivo* at nano/micro second levels, we require higher speed without compromising the resolution and without generating unwanted artifacts and stray noise interference. A1 and A1R goes well with the recently introduced Ti-E inverted microscope system (figure 1), especially when coupled with Nikon's patented 870nm Perfect Focus System (PFS) that ensures the elimination of

focal drift though IR based hardware control. Due to high optical efficiency and 16 million pixels resolution, high quality confocal images can be achieved that will bring inter/intra cellular nuance into the limelight. Along with the Ti-E inverted microscope, the A1/A1R confocal system set a new standard for advanced time-lapse molecular imaging of rapid cellular interactions to bring biological imaging to life. A1 and A1R are separate products and there is no possibility to upgrade A1 into A1R confocal system.

Nikon claims that the new system can resolve rapid biological events with ultra high resolution. This evolved version of the present real time spectral confocal system

C1si, have other unique features like diffraction efficiency enhancement system based multiple gratings (DEES), weak signal sensitivity through dual integration signal processing (DISP), pre-calibrated synchronized 32 channel multi-anode PMTs, high-efficiency fluorescence transmission technology to achieve high optical transmission and most importantly the faster spectral unmixing algorithm that enables high speed spectral imaging without any molecular crosstalk in real time. DISP enhances the sensitivity by using a pair of integrating digitizers in order to assure the data is gathered over the full pixel period without any down time delay. Together with these



Figure 1. A1/A1R Confocal system integrates motorized inverted microscope, hybrid dual scan head for high speed as well as high resolution scanning, 4 channel fluorescence detector, 32 channel spectral detector

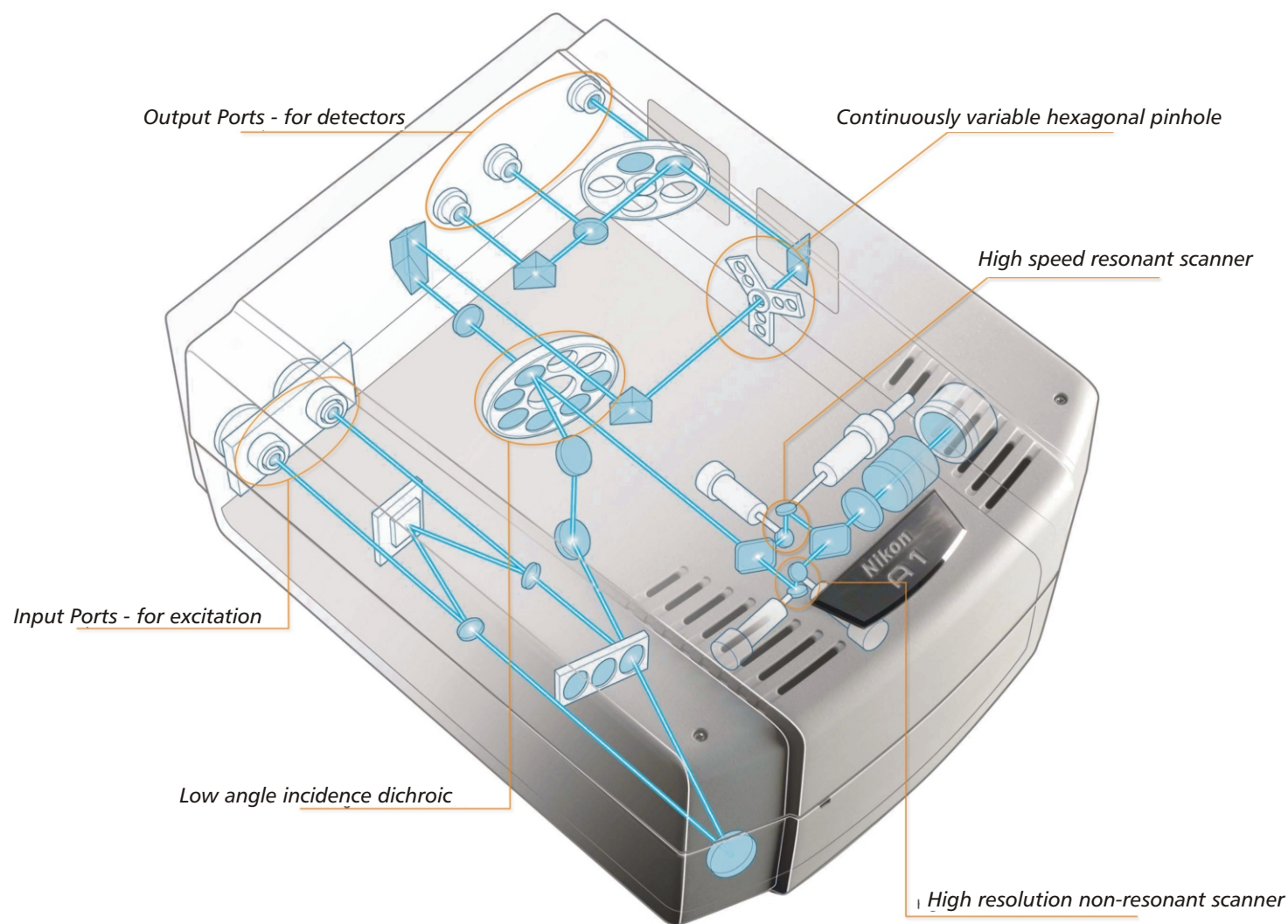


Figure 2. The scan head architecture includes innovative design features like low angle incidence dichroic mirror; hybrid scanner and continuously variable hexagonal pinhole. There are three output ports to allow optical fiber connection to three separate detector units. Opening the pinhole in fiber coupled spectral detection system avoids the loss of spectral resolution and spillover into adjacent channels.

technical features, A1R confocal system comes with high resolution and high speed hybrid scanner, low incidence angle dichroic mirror, hexagonal continuously variable pinhole and an innovative virtual adaptable aperture system for deconvolved simultaneous image acquisition both at focal and non-focal plane with a single scan with higher speed and reduced cell damage.

### Scan head design features

The A1 and A1R systems are in accordance with the concept of the previous C1 family confocal system. The optical fiber connection evade the influence of heat in the scan head as

the heat may cause misalignment of optical components and interfere with the sensitivity of the scan head. This avoids unwanted artifacts that may lead to false positive and false negative conclusions while analyzing the confocal images. The A1 system has standard paired galvanometers that gives high resolution images up to 4K x 4K pixels, whereas A1R incorporates two independent galvo systems high speed resonant and high resolution non-resonant hybrid scanner system that offers the speed of 30 frames per second at 512 x 512 pixels (figure 2). The resonant scanner has a frequency of 7.8 KHz (is mounted along with the non-resonant scanner) and gives industry's fastest 230 fps at

512 x 64 pixels facilitating ultra-high-speed imaging without compromising image quality. The fiber-optic communication data transfer system can transmit data at a maximum of 4Gb/sec. that allows transfer and recording of image data at 512 x 512 pixels in five modes at more than 30 fps.

### Scanning mode and performance

A1R scans X-axis through resonant galvanometer with the resonance frequency of 7.8 KHz and Y-axis through non-resonant scanner with seven steps (1x, 1.5x, 2x, 3x, 4x, 6x and 8x) scanner zoom achieving 512 x 512

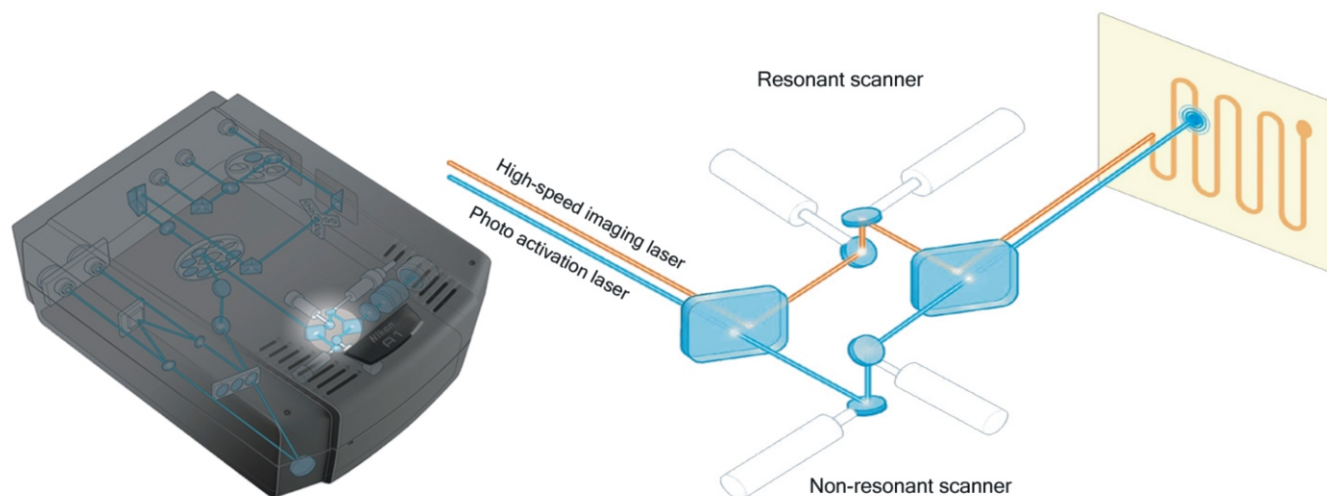


Figure 3. AIR system integrates both resonant high speed as well as non-resonant high resolution scanners. The spot positions of resonant scanner and non-resonant scanner are different, hence ensuring no light cross-talk. Flexible switching or simultaneous use of both the scanners is possible with high-speed hyper selector that allows ultrafast imaging and photo activation imaging required in cell dynamics and molecular interaction studies.

maximum pixels, whereas A1 XY scanning is through non-resonant galvanometer with 1 to 1000x continuously variable scanner zoom that generates 4096 x 4096 pixels high resolution imaging. Scanning can be done via three modes either by using the resonant scanner alone for high speed imaging up to 230 fps, by using the non-resonant scanner alone for high resolution imaging up to 4K x 4K pixels or by combining both resonant scanner and non-resonant scanner for simultaneous photo-stimulation imaging (figure 3). The scanners are used in tandem that enables the resonant scanner to capture the images at 30fps at the same time, when the non-resonant scanner photoactivates or photobleaches the specimen.

Scanning speed steps are variable with 0.48 sec of dwelling time step, ensuring low photo-bleaching and photo-toxicity with enhanced cell viability. The high speed hyper-selector allows flexible switching as well as simultaneous use of both the galvo scanners that enables flexible operations for a wide range of applications. This mechanism enables simultaneous photo-activation and imaging with improved sensitivity and reduced photo-bleaching and photo-toxicity, which is vital for most of the sensitive functional cell dynamics and molecular interaction applications and in turn reveals

high temporal and spatial resolution of intermolecular interaction. The optical pixel clock generator that is used to generate ultra stable clock sync pulses, produce images which are completely even in intensity without any distortion at high speed. The hybrid scanning system also allows high speed imaging up to 420fps (2.4ms/frame) at 512 x 32 pixels. This supports advanced live cell imaging works and molecular cell dynamics applications like photo-activation, photo-conversion, FRET, FRAP, FLIP and FLIM in a more efficient way. The system comes with the analysis software for FRAP and FRET as standard.

### Laser system, control and CLEM

There are three beam introduction ports that allow the connection of two fiber coupled laser sets and one air space coupled laser. Two laser input ports are incorporated in the scan head to use different laser lines. The AOTF modulated 4 laser unit is used as a standard that provides 7 laser lines (choices from 405nm, 440nm, 457nm, 488nm, 514nm, 543nm, 562nm and 638nm) and the AOM modulated 3 laser board can be added as an option that provides additional 3 laser lines, hence 7 lasers with 9 lines are available at maximum. In addition, it has an optional

picosecond or faster IR pulsed laser port as an option. Laser power is controlled by the absolute value not through the transmission of AOTF and they are modulated through power control for each wavelength, return mask and ROI exposure control. Through the software along with AOTF and AOM, the lasers can be controlled in the unit of 0.1%. In addition, software variable control with continuous ND is also possible.

The input ports are continuously monitored for the laser power that is governed by the control system, ensures quantitative and uniform performance. The current trend in confocal specimen analysis is the simultaneous observation of four color stained sample such as 405 excitation for nucleus stained by DAPI or Hoechst, 488 excitation for GFP or FITC, 561 excitation for Rhodamine or Cy3 and 638 excitation for Cy5 or Alexa647. The 4 laser unit has developed to meet such demanding application with accuracy. An additional advantage of the A1/A1R system is its ability to be used in combination with Controlled Light Exposure Microscopy (CLEM) that was developed by Erik Manders and his team at the University of Amsterdam for automatically monitoring and varying laser illumination during time-lapse studies in order to minimize the risk of laser induced biochemical inconsistency, cell





Figure 4. With conventional dichroic system, reflection-transmission characteristics have high polarization dependence. With the new low angle incidence method, reflection-transmission characteristics have lower polarization dependence.

degradation, death and photo-bleaching. CLEM reduces laser illumination by sensing the resulting fluorescence and using sub-microsecond feedback to adjust the incident laser light on the specimen.

### Low angle incidence dichroic mirror

The industry's first low incidence 12° angle excitation dichroic mirror enhances 30% more fluorescence efficiency and 99% transmission in combination with high performance sputtering as the reflection-transmission characteristics have lower polarization dependence, when compared to conventional incidence angle (45°) method, where reflection-transmission characteristics have high polarization dependence (figure 4). The emission filters and dichroic mirrors are sputter coated that assures high throughput and outstanding channel separation. The emission dichroic mirror has the capability of mounting 18 types simultaneously (6 types of dichroic blocks x 3 wheels). Users can switch the blocks without adjustment. The eight position dichroic mirror has the standard filters for different wavelength range (405/488, 405/488/561, 405/488/561/638, 405/488/543/638, 457/514 and BS20/80).

### Continuously variable hexagonal pinhole

Ideally, the pinhole shape should be circular. With the A1 system, the continuously variable hexagonal pinhole (12-256  $\mu$ m), which

replaces the standard four-sided aperture, considerably sharpens the image quality and allows 30% more light resulting in brighter images with the same sectioning performance. Since the area of hexagon inscribing a circle is bigger than the area of square inscribing the same circle, the hexagonal pinhole becomes a better approximation with respect to the ideal circular pinhole. When a square pinhole opens up to change the diameter, it lets through more light but the resolution is greatly challenged and ebbed out from the interest of the experiment. Thus with the innovative hexagonal pinhole design, maximum confocality is maintained while achieving higher brightness equivalent to that of an ideal circular pinhole. The single pinhole system design for all channels assures perfect registration between channels. Opening the pinhole in non-fiber coupled spectral detectors cause loss of spectral resolution and spectral spillover into adjacent channels that may generate possible artifacts. Since, A1 system is coupled with optical fiber connection, there is no spectrum wavelength resolution change during the pinhole opening which is so unique in its kind.

### Virtual Adaptable Aperture System (VAAS)

Increasing the pinhole size may generate brighter images but with blurred resolution due to haze and pinhole induced distortions. Reducing the pinhole size, to eliminate flare light from non-focal plane results in unblurred

but darker images. Virtual Adaptable Aperture System (VAAS) pinhole unit provides better images with less flare as the light that a confocal pinhole rejects is collected by another detector. VAAS signals are collected by subtracting part of the light reflected from the peripheral area of the pinhole. The light that passes through the pinhole has focused light but little out of focus light, whereas the light that reflects from the peripheral area of the pinhole is out of focus that has some focus light. The main idea of VAAS is to collect the required light signals that are passing through the pinhole as well as the needed signals that go out of pinhole.

It allows simulation of different sectionings and slice thicknesses after image acquisition and has a better control over the attainment of experimental data. VAAS can also provide post acquisition recovery of photon data that are normally lost due to the physical pinhole size during the course of the experiment. This unique detector system allows virtual adjustment of the confocality and sensitivity by collecting more photons during the initial image acquisition to generate a high resolution image and presumably a more sensitive one. NC-VAAS is reducing the haze and the PC-VAAS is used to change pinhole diameter virtually. VAAS detection is an upgrade option that is expected in October 2008.

### Detector system

The newly fully automated standard fluorescence detector with 4 PMTs facilitate to acquire 4 color images simultaneously in the range of 400 nm to 750 nm (figure 5). This detector unit has changeable filters that allow simple onsite installation of emission filter and mirror sets. Six emission filters are used on three filter wheels. In combination with four lasers, simultaneous observation of four different fluorescence labels is possible. Image quality is further enhanced by hyper double sampling, wherein the PMT noise is sampled twice and eliminated hence reducing noise in images. The diascopic PMT detector for transmitted light works with the wavelength range of 440 nm to 700 nm.

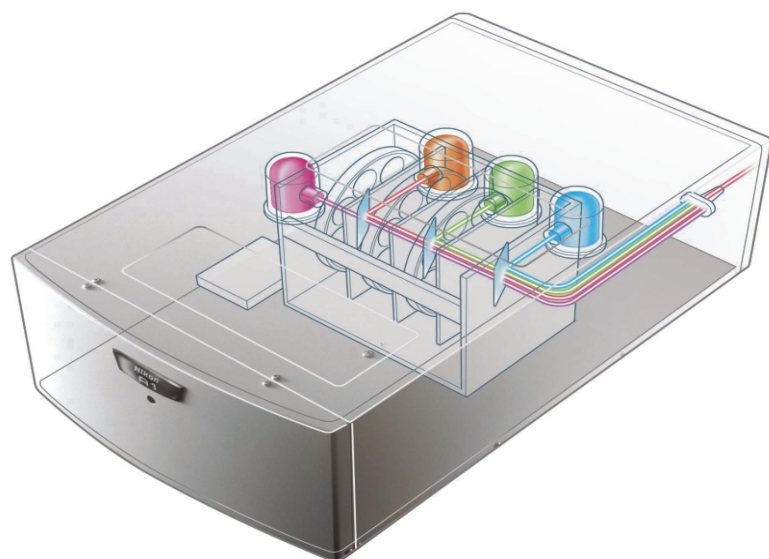


Figure 5. In combination with four lasers, simultaneous observation of four fluorescence labels is possible with 4 PMTs in the wavelength range of 400 nm to 750 nm

### Spectral detection, unmixing and V-filtering

When compared to C1si confocal system, the spectral detection performance of A1 is enhanced further along with V-filtering function that expands the range of use of spectral images. Using V-filtering function, up to four preferred spectral ranges can be chosen from 32 channels and the intensity of each range can be adjusted independently and this allows selection of desired spectral range and flexibility to handle new fluorescence probes (figure 6). Together with the high speed AD conversion circuit, the new signal processing technology allows simultaneous 32 channel spectral image acquisition at 512 x

512 pixels in 0.5 seconds. At 512 x 64 pixels, images can be acquired with the speed of 16 frames per second.

The linear high speed and high accuracy unmixing algorithm and high speed data processing enable fast and accurate unmixing during image acquisition in less than a second. When coupled with high speed spectral imaging, an image devoid of autofluorescence and molecular crosstalk can be created in real time that gives true spectral imaging experience. User friendly laser shields allow simultaneous use of four lasers and enable nine colors with broader band spectral imaging. There are also three output ports to allow optical fiber connection to three separate detector units like spectral detector

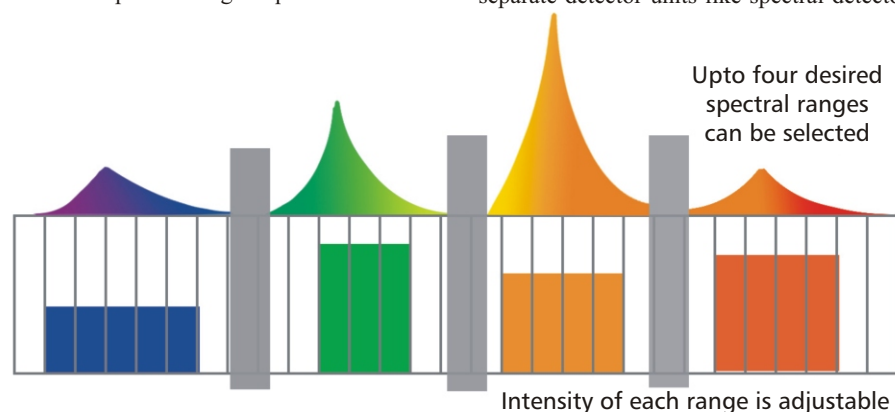


Figure 6. V-filtering function enables up to four desired spectral ranges that can be selected from 32 channels. Total intensity of each range can be adjusted individually similar to separating colors and controlling four PMTs by using optical filters that allows acquisition of the desired spectral range and providing flexibility to handle any new fluorescence probes. (Graphical representation is only illustrative not as per scale).

unit and custom detectors for FCS or FLIM. Specifically, the A1/A1R system features a new optional spectral detector for concurrent acquisition of up to 32 channels. Three spectral resolutions or channel widths are available (2.5nm, 6nm and 10nm), simultaneously covering up to 320nm of spectrum at each frame scan. If spectral sensitivity is a greater concern than axial resolution, the pinhole can be opened without generating any artifacts because it is the emission fiber core that is imaged on the detector, not the pinhole itself.

### Conclusion

The stringent live cell imaging applications requires specific technical design and configuration of Lazer Scanning Confocal Microscope, which has to be placid and serene on the developing and proliferating cells. Together with innovative technical features like low angle incidence dichroic mirror, continuously variable hexagonal pinhole, hybrid scanner and VAAS the A1/A1R confocal system appears to be an efficient system. The limitations with the existing confocal microscopes like performing real time spectral confocal, live co-localization studies, effective high accuracy spectral unmixing to avoid molecular cross talking, long time dynamic live cell imaging, focus drift correction in live cell analysis, weak signal capturing and autofluorescence correction can be effectively addressed by A1/A1R systems that may open up new vistas in understanding the molecular intricacies of the cellular processes.

#### About the Authors

**Vee-Jay Light** and **Gene Maverick** are working for Nikon Bio-science division. The views, opinion and ideas expressed here do not reflect to the organization they belong. Though the authors tried to focus on the technology behind the system, there may be a possible commercial interest with this article but there is no conflict of interest.

E-mail: [genemaverick@technologist.com](mailto:genemaverick@technologist.com)