Spinning Disk Microscopy

11

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What You Learn from This Chapter

Spinning disk microscopy is a specialized imaging technique utilized with living and light sensitive samples. Arrays of optical pinholes spun at high speeds are used to focus the excitation source and block unfocused emitted fluorescence to derive confocal images.

11.1 Overview

Much of biomedical research is ultimately dependent on using live-cell imaging techniques. This requirement has fueled remarkable advances in microscopy instrumentation as well as the development of state-of-the-art detection systems and fluorescent proteins. The imaging of living samples poses several complications not necessarily seen in fixed tissues or other non-living specimens. Life is neither

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two-dimensional nor is it simple. Therefore, to study life while it is alive, we need to do so in a three-dimensional fashion which accommodates for the idiosyncrasies of keeping the environment sustainable for the specimen.

There is an innate thickness and heterogeneity which affects the resultant image: a lipid membrane clouds the surface of the cell, internal organelles of differing shape, size, and optical density all refract incident light, and specific proteins are not visible without contrast. To study that cell while it still living adds another layer of complexity: vesicles and other sub-cellular structures are in constant motion, adding the contrast needed to visualize sub-cellular attributes is commonly toxic, and the necessary high energy light sources are phototoxic to living cells. A single image cannot capture movement; a single image cannot focus in multiple focal plans simultaneously, and a single image cannot provide enough detail to study the aspects of a cell which make it live. Spinning disk microscopy alleviates these issues.

For the highest of imaging specificities, collimated excitations sources, such as lasers, are further focused through the system's optical pathway. Initially, the beam passes through a disk which is made up of a series of microlenses to focus the beam through a dichroic mirror and onto the pinholes in the second (Nipkow) disk. After passing through the Nipkow disk, the process is like standard fluorescent microscopy as the excitatory beam then passes through the chosen objective and onto the sample being observed. The emitted signal from the specimen is gathered by the objective lens, filtered back through the chosen dichroic mirror, and sent back through the pinholes of the Nipkow disk, filtered through the chosen dichroic mirror and directed toward the camera port for detection.

The entire specimen is scanned by sets of multiple pinholes as the disk spins across the sample leading to images of the focal plane being taken in rapid succession. Using this form of array scanning allows for high spatial and temporal resolution while limiting the phototoxic effects of intense laser stimulation. The technique allows for imaging on a microsecond timescale which is reflective of many biological processes, while doing so in an environment which is much less detrimental to the processes themselves.

11.2 History

Originally developed for use in televisions, Paul Nipkow's design to spin a disk comprised of identical equidistant pinholes for image reconstruction was patented in 1885 [1]. Future iterations of the disk and scanning device were then incorporated into microscopy, most notably with the help of Mojmir Petráň.

Unlike the original Nipkow disk, the Petráň disk (see Fig. 11.1) was constructed with many Archimedean spirals embedded in the disk, which allows for multiple point illumination and detection, leading to the development of what we now know as spinning disk confocal microscopy. Nesting the Archimedean spirals allows for hundreds of pinholes to be utilized on a disk, hence drastically increasing the light throughput to the specimen.

An Archimedean spiral pinhole pattern is derived from the pinholes being placed in a stepwise fashion along an imaginary line on the disk which rotates around the center to the outermost edge creating an arc of 360 degrees. Imagine if the disk were segmented into concentric circles as well as segmented into equal "pie" portions with radial lines. Starting at the origin, in a clockwise fashion, a pinhole is added where the first radial line intersects the first concentric ring, then the second, and the third. As long as the number of "pie" segments and the number of radial lines are equivalent, the spiral's last radial line will intersect the outermost ring (or edge of the disk area) after a full rotation. See Fig. 11.2.

In the microscope systems, the disk is spun at a constant speed around the origin which provides a constant angular momentum. The distance between the pinholes is also constant by following the spiral pattern. By nesting several spiral pinhole patterns on a single disk, the entirety of the specimen can be scanned very quickly as the disk rotates. Commonly in spinning disk microscopy systems, the disk is segmented into 12 sections each equaling 30° of the 360° disk circumference. These original spin disk systems utilized light sources that were powerful for the time, such as arc lamps. The broad-spectrum incident light would pass through the pinholes and be directed to the specimen. The resultant signal then is filtered for the desired wavelengths through a beam splitter, routed off several mirrors and through the pinholes on the opposite side of the disk where scattered light is limited.

The light path was complicated which made fine-tuning the system to an experimental need difficult. At the time, the added light redirections were necessary to limit glare and reflections from the components of the system itself, such as light bouncing off the metal disk. Further iterations of the spinning disk microscopy systems limited the internal reflections of signal through a variety of ways to allow more efficient light paths to emerge.

Modern spinning disk systems employ technologically advanced disks, light paths, and light sources which are only reminiscent of these early microscopes.

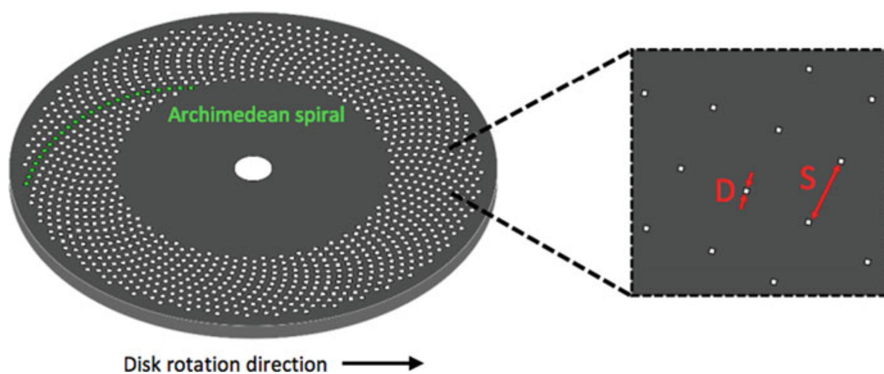


Fig. 11.1 A standard Nipkow–Petráň disk. The pinholes are arranged in Archimedean spirals and have a set diameter (D) and separation distance (S). Alteration in disk spin speed, (D), and/or (S) the image brightness, contrast, and quality can be optimized for an experiment [2]

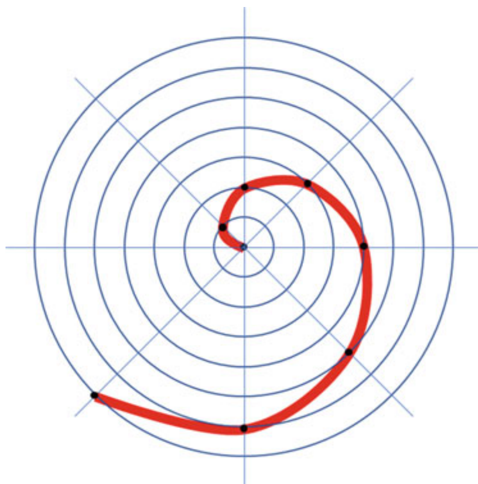


Fig. 11.2 The Archimedean spiral

Prevailing terminology often refers to Nipkow or Petráň in reference to any spinning disk microscopy system, regardless of the disk structure actually being used.

11.3 Sample Preparation

As with most scientific endeavors, preparation is of the utmost importance. To the naked eye, an animal cell under standard visible light provides little to no detail. The bulk of a cell's content is water, which is nearly transparent. Some gross cellular structures are discernable, at best. In order to view any detail with specificity, contrast must be added to the sample in the desired locations of study. As covered in the previous chapters of this textbook, standard fluorescent microscopy most commonly utilizes antibodies to target subcellular structures. In living cells, however, that procedure is not useable as it requires the cell to be fixed in place and the membranes damaged, which would kill a living cell. Two of the more common techniques for imparting contrast for in vivo imaging are (1) the use of an expressor which has been genetically introduced to a cell through transfection, such as GFP, or (2) the use of vital dyes, more commonly known as probes, which can be utilized to target specific subcellular compartments (such as organelles), or specific cellular processes (such as reactive oxygen species production, membrane potential, calcium presence, and pH gradients) [3].

Not only must the chosen probes be targeted to what one wants to image, they must also be compatible with each other, having little or no spectral overlap. Spectral overlap occurs when two (or more) fluorophores either excite or emit in the same spectral range. This is especially problematic in a spinning disk system as the cameras used to capture emitted photons are monochromatic and unfiltered to ensure the highest sensitivity. Therefore, if multiple probes are used in the same sample,

they are in need of being imaged completely separately to prevent contamination of the signals.

Beyond compatibility with life, and compatibility with each other, the probes must also be imageable with your microscopy system [4]. There are a wide variety of spin disk systems available, most of which can be tailored to specific imaging needs. If a system has a white light excitation source, it can excite multiple fluorophores, but would likely do so simultaneously without sophisticated filtering systems. A system with a single wavelength laser while exciting only the desired fluorophore would limit which fluorophores are useable in an experiment. And if the fluorophore you wish to use only emits in the far-red spectrum, having a camera that is not sensitive at those wavelengths will not permit data accumulation from the experiment as designed. The need for multiple laser lines and high-end cameras, along with proper computational and distinctively elegant software control systems, can lead to overwhelming system costs which often make it impossible for a single investigator to own all of the necessary equipment.

Once a specified cellular process or subcellular target is identified and properly labeled with a probe, and the microscope is set up to equip the desired experiment, then one must also consider keeping the sample alive to do the imaging. Living cells require the maintenance of proper oxygen/carbon dioxide balance, temperatures, nutrients, and humidity to maintain proper functions. Common scientific accessories can balance these provisions readily, but they need to be tailored to work on a microscopy system.

Experimental design is crucial to a favorable experimental outcome. Proper probe choices, proper microscopy configuration, and proper imaging conditions must all be taken into consideration. What seems to be a simple experiment can become a delicate balance with multiple caveats which must simultaneously culminate at the exact time an investigator has access to use the needed microscope.

11.4 Fundamental Microscope Design

Attaining confocality requires tremendous incident energy to attain a limited fluorescent signal from a fairly dark specimen. This requires the unused incident light, and reflections thereof, to be eliminated from the detection pathway along with any light which becomes scattered by passing through the system, disk, or sample itself. This requires a unique light path since there is the inclusion of moving parts, i.e., the spinning disk.

At the time this is written, Yokogawa Electric and their Confocal Scanning Unit (*CSU*) are leading the commercial production of spinning disk microscopy systems. These scanners consist of two aligned disks with a dichromatic mirror positioned between them (Fig. 11.3). This allows the incident light to be filtered between both disks, while the emitted signal also passes through pinholes to further filter out-of-focus light. The upper disk contains microlenses in the pinholes to focus the incident light onto the pinholes of the lower disk (Fig. 11.4). This allows for greater efficiency of incident excitation energy reaching the sample, allowing for lower energies to be

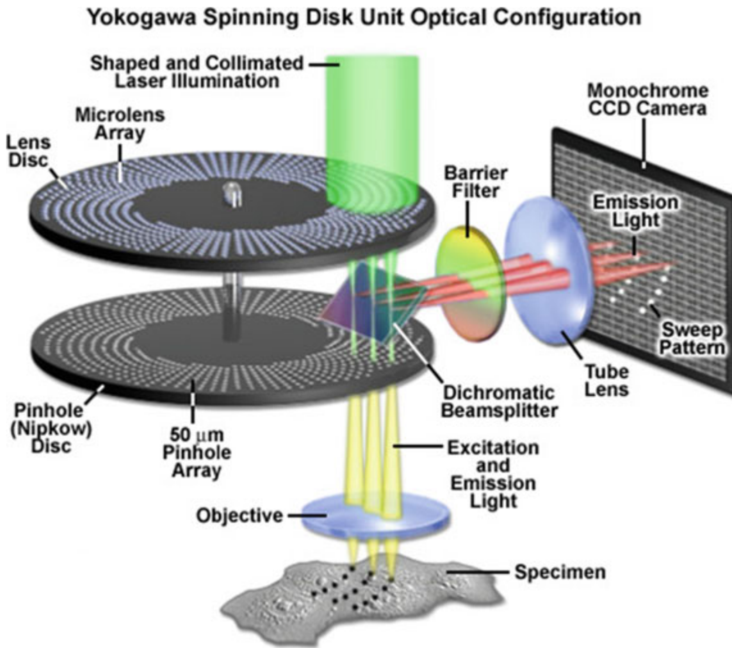
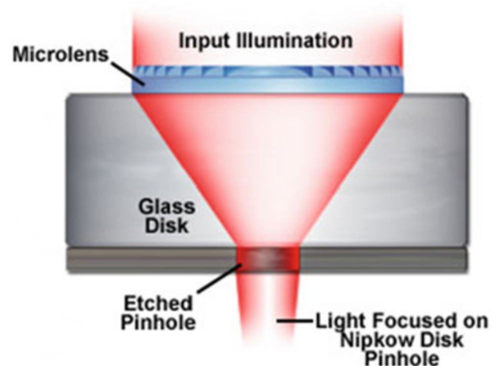


Fig. 11.3 Schematic illustration of the Yokogawa CSU-X1 spinning disk confocal optical pathway [5]

Fig. 11.4 Microlens focusing incident light toward pinhole in Nipkow disk [6]



used and benefits such as lower phototoxicity rates for living specimens and a greater ability to produce a signal from specimens which may only be expressing low levels of a fluorescent reporter.

These spinning disk scanners have rotation speeds of up to 10,000 revolutions per minute, and a pinhole array pattern scanning 12 frames per rotation. This creates a theoretical imaging speed up to 2000 frames per second.

$$10,000\,\frac{\text{revs}}{\text{min}} * 12\,\frac{\text{frames}}{\text{rev}} * \frac{1\,\text{min}}{60\,\text{sec}} = \frac{2,000\text{frames}}{\text{sec}}.$$

Camera sensitivity is paramount in attaining sufficient emitted signal and therefore required exposure time often becomes the limiting factor in speed of image acquisition over disk RPM. With a low-level fluorescence emitting sample, exposure rates can easily slow acquisitions from thousands of frames per second to single digits.

Following the light path of the scanner helps the understanding of how confocality is achieved (Fig. 11.3). Laser light (green) is collimated and projected onto the top disk, which is made up of microlenses. Each microlens (Fig. 11.4) focuses the gathered light through a dichromatic beamsplitter onto a section of the lower/Nipkow type disk.

After being focused through the two disks, the incident light from each pinhole is focused through the back aperture of the objective through to the specimen where it triggers the fluorescent signal emission in the plane of focus. The fluorescent emission then is gathered through the same objective, where it travels back up through the Nipkow type disk. The pinholes in the Nipkow type disk act as confocal apertures, blocking out-of-focus light. The emitted signal passes through the same pinhole as the excitation light which eluded the signal. It then passes to the dichromatic beamsplitter. The beamsplitter allows short wavelengths (excitation) to pass through while reflecting long wavelengths (emission) through a barrier filter which eliminates excess and non-wavelength-specific light. It then continues the path toward the camera for detection.

In order to attain accurate and useful images from a spinning disk microscopy system, the speed of the disk needs to coordinate with the camera acquisition rate, which is dependent on the emitted fluorescence of the sample. With there being 12 imaging frames per revolution and 360° in a revolution, one can easily conclude that each imaging frame occurs during a disk rotation of 30°. Since a complete image of the sample is scanned every time the Nipkow disk rotates 30°, the camera acquisition time has to be based on the time it takes for the disk to rotate that 30° or else distortions will occur. If the image acquisition time is set longer than the time for (or a multiple of the time for) a 30° disk rotation, then the disk and pinhole will be visible in the attained image, appearing as stripes or lines (Fig. 11.5c).

Detecting low light at high speed in a specimen that has moving parts requires extremely sophisticated camera systems. Slow acquisition low quantum efficient photomultipliers are insufficient for this level of imaging. Scientific CMOS cameras capture light so efficiently that low photon producing specimens can be imaged down to the single molecule level. Once the camera's sensor is contacted by a photon emitted by the specimen, the signal is amplified prior to read-out which greatly enhanced the signal-to-noise ratio (essentially the contrast of the image). This is ideal for spinning disk microscopy as the signals produced are most commonly extremely weak due to the need to have fast imaging rates with minimal exposure and low

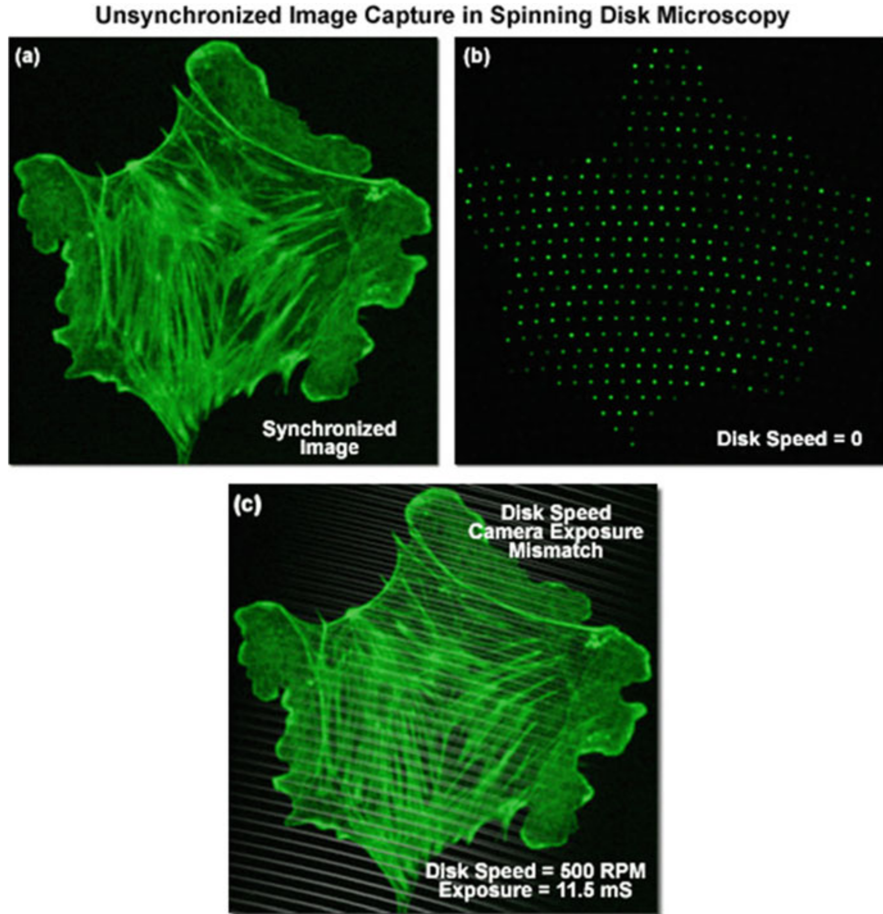


Fig. 11.5 Image comparison from (**a**) camera and disk rotation speed are synchronized, (**b**) camera image when the disk is not in motion, (**c**) visible banding from exposure/disk speed mismatch [5, 6]

excitation energies to avoid phototoxicity. The amplification comes from an additional read-out register. The EM register uses high voltages to add energy to the incident electrons allowing impact ionization; create an additional electro–hole pair and hence a free electron charge which is stored in the subsequent pixel and hence increasing the signal-to-noise ratio. Of course, signal amplification can also mean noise amplification. The electron multiplication will amplify any incident photon signal regardless of origin.

Scientific CMOS cameras are currently gaining sensitivity and have the speed to capture images in real time, aiding biological researchers' understanding of live-cell interactions. CMOS cameras bring the benefit of high frame rate and wide fields of

view for imaging faster moving samples.. Choosing the correct camera for the desired experiment is imperative in attaining the best results possible.

11.5 Imaging Resolution

As shown in Fig. 11.1, the pinholes in a Nipkow disk have a set diameter (D) and separation distance (S) between pinholes which is quite a bit larger than the pinhole diameter. While any reflected light from the focal point is gathered through the pinhole, the inter-pinhole disk space physically blocks out-of-focus reflected light, permitting confocality. The smaller the pinhole diameter, the higher the confocality, but a trade-off occurs because less light can be gathered from the sample. In a very bright sample, this may not be an issue; however, live-cell imaging most commonly involves low-level fluorescence along with cellular background autofluorescence leading to a much smaller signal-to-noise ratio than can usually be attained in a fixed and/or stained tissue sample. Optimizing image acquisition occurs not only by synchronizing the camera acquisition speed to the disk rotation speed, but also by using a disk with optimized pinhole diameter and spacing for the highest available brightness and contrast [2].

So how can you tell what the optimized pinhole diameter and spacing would be? Similar to other forms of microscopy, the lateral resolution is directly related to the excitation wavelength and the objective being used. The Abbe equation for spatial resolution depicts this relationship:

$$d = 0.61\left(\frac{\lambda}{NA}\right).$$

Setting up an experiment with a 100× 1.4 *NA* objective and using a common wavelength green laser light of approximately 488 nm, the diffraction limit of the imaging system would be approximately 212 nm. The same objective being used with a white light source (300–700 nm) would provide an Abbe resolution closer to 305 nm. If the disk being used for the experiment has pinholes smaller than the resolving power based on the Abbe equation, the resultant images will be compromised since too much of the signal will be blocked. Most spinning disk systems will have interchangeable disks with pinholes of varying sizes to allow for this optimization. Disks with 25 μm and 50 μm and 70 μm are commonly available.

Pinhole size is only part of the optical optimization. Confocality can only be achieved by blocking the out-of-focus light, which mainly comes from scattering as the incident or reflected light passes through the sample. The pinholes must be placed far enough apart that scattered light from the emission signal of one pinhole does not reach an adjacent pinhole. Ensuring the proper pinhole size and distribution on the disk along with the previously discussed microscopy factors such as wavelength of excitation light, objective numerical aperture, refractive index of

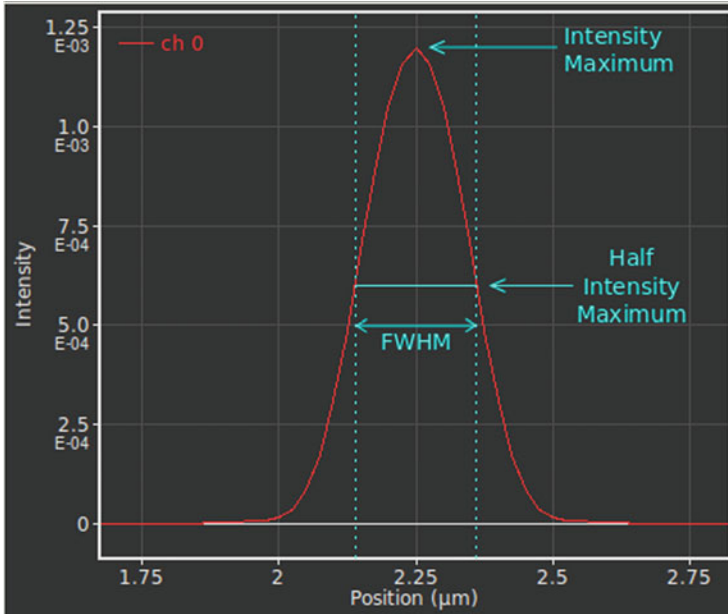


Fig. 11.6 Full width at half maximum (FWHM) graphical representation of a point-spread function intensity plot [7]

immersion medium, and camera functionality are all important factors in attaining quality images with high axial resolution.

Living processes rarely occur solely in a lateral format, however. Three-dimensional organisms have three-dimensional processes which need to be studied in three-dimensions. This is where optical sectioning is employed. A specimen can be scanned through focal planes in the Z-direction. The thickness of each focal plane is determined by the point-spread function (PSFThe PSF is a measure of how a microscope under the experimental parameters images a single point of signal, most commonly a fluorescent bead of a specified size. Laterally, the bead may appear circular and distinct; however when the bead is imaged through a depth there will be a distinctive blurring.

Since biological samples are nonhomogeneous, the emitted signal is refracted in multiple ways while passing through. The refractive index of a cell membrane is different than the refractive index of the intracellular fluids, which is different from the refractive index of neighboring organelles, all of which affect the scattering of the desired signal before it even reached the microscope. The blurring in the PSF in a transparent gel versus the blurring of the PSF in the chosen live sample can be used to mathematically deconvolute the images post-acquisition.

To determine the axial resolution of the microscopy setup, the full width at half maximum (FWHM) of the point-spread function is used. As seen in the PSF intensity plot in Fig. 11.6, this is quite literally the full distance where the intensity of a fluorescent object is half of the most intensely fluorescent point.

The FWHM can be tested experimentally, as with imaging a fluorescent bead. It can also be calculated using the equation:

$$\text{Axial resolution} = \sqrt{\frac{0.88\,\lambda_{\text{exc}}}{\left(n - \sqrt{n^2 - NA^2}\right)} + \left(\frac{D * n * \sqrt{2}}{NA}\right)^2},$$

where λ_{exc} is the excitation wavelength, n is the refractive index of the immersion medium, and NA is the objective numerical aperture and D is the pinhole diameter [8]. A 100× 1.4 NA oil immersion objective using a Nipkow disk with 50 μm diameter pinholes and a laser excitation of 488 nm would lead to an axial resolution, or FWHM, of almost 900 nm. Imaging optical sections less than 900 nm in this scenario would not yield any higher quality image but would overexpose the sample to laser intensity and hold a higher risk of photobleaching.

The pinhole in a spinning disk microscope is not adjustable and often is the limiting factor in resolution when considering faint or low signal-to-noise ratio samples, as is common in living specimens. If a system only has a single disk and imaging produces an image smaller than the pinhole, a tube lens can be inserted into the light path to magnify the image as needed. If the produced image is larger than the pinhole, part of the desired signal will be blocked by the pinhole aperture and lost.

Calculating the resolution of a spinning disk microscopy system involves a variety of factors which are both innate to a specimen and innate to the imaging system. Signal mitigating factors such as objective magnification, objective immersion fluid, excitation source, pinhole size, and pinhole separation all have to be taken into account to optimize images.

11.6 Super Resolution via Optical Reassignment Imaging (a.k.a. SoRa)

While standard imaging and even advanced techniques like spinning disk microscopy allow the discovery and exploration in living organisms, the constraints are imparted by the systems' technology creating unsurpassable thresholds; resolution being a drastically limiting factor. Conventional fluorescence microscopy alone is unable to resolve structures less than 200 nm in size. While some organelles in a cell could be visualized, many are below the 200 nm threshold, with proteins being smaller still. In a dormant sample, some super-resolution microscopy can be utilized to break through this imaging barrier. However, living samples are not only sensitive to the phototoxicity resultant from such techniques, but also the timescale of living interactions often occurs at a rate which cannot be captured using standard super-resolution techniques [9]. Theoretically, spinning disk super-resolution microscopy can achieve imaging at 120 nm and do so with a temporal resolution suitable for measuring the rapid dynamics of biological samples [10].

Imaging beyond the diffraction limit is necessary for the further study of living organisms, as many organisms and cellular processes occur at this scale. In order to overcome the barriers of phototoxicity and artifacts from processing, optical reassignment can be used. While extremely complicated to do well, the concept of optical reassignment is quite elegant, and in an oversimplified explanation, essentially equates to adding an additional lens in the emission pathway [11]. Microlenses can also be added to the pinholes in the Nipkow disk [12]. Adding the lens optimizes the light which has passed through the pinhole of the Nipkow disk, to fill the pupil of the objective, maximizing function. The emission signal is then collected as it passes through the lenses in the emission pathway and is optically contracted before reaching the detecting camera system.

Filtering of the acquired image must also occur to reach super-resolution levels. Traditional mathematical algorithms can be applied to images to filter out blur and attempt to resolve objects below the resolution limit of a system. Commonly, filtering algorithms create statistical estimates of an image using a known and measured standard, such as a point-spread function. There is an inherent danger in that these algorithms commonly impart artifact images derived from living samples due to the fact that the algorithms assume the sample and sample noise are stationary [13]. When focusing on living samples, virtually nothing is stationary. To limit artifact production from filtering, algorithms which specifically amplify high spatial frequencies over the low spatial frequencies are used. This creates a focusing of the deconvolution where the highest level of signal is, lessens the artifacts which can develop near the actual signal, and creates a more reliable data set [14] (Fig. 11.7).

11.7 Spinning Disk Confocal Microscopy vs. Laser Scanning Confocal Microscopy

There are other forms of confocal imaging. Laser scanning confocal systems have some similarities to spinning disk confocal systems. Both system types are used to image at a microscopic level in high detail. Both utilize a pinhole system to limit or eliminate out-of-focus light for detailed examination of a specimen. Both utilize or can utilize lasers for specific fluorophore excitation. However, each system is unique in the type of specimens imaged and desired data output.

By utilizing specialized cameras with a high quantum efficiency instead of photomultiplier tubes, faint signals can be accurately detected. This is important for multiple reasons. Firstly, many of the fluorescent dyes used in microscopy are somewhat toxic to living organisms. The less dye which can be introduced to the system, the healthier and less altered the living sample remains. Additionally, the utilization of so many confocal pinholes in a single disk allows for large segments of a specimen to be imaged simultaneously, unlike the raster scan of a laser scanning confocal, which has a single pinhole dragging back and forth across the imaging area. There is nothing static about living processes. Waiting for a pinhole to scan from one corner of an imaging area to the next would provide limited information

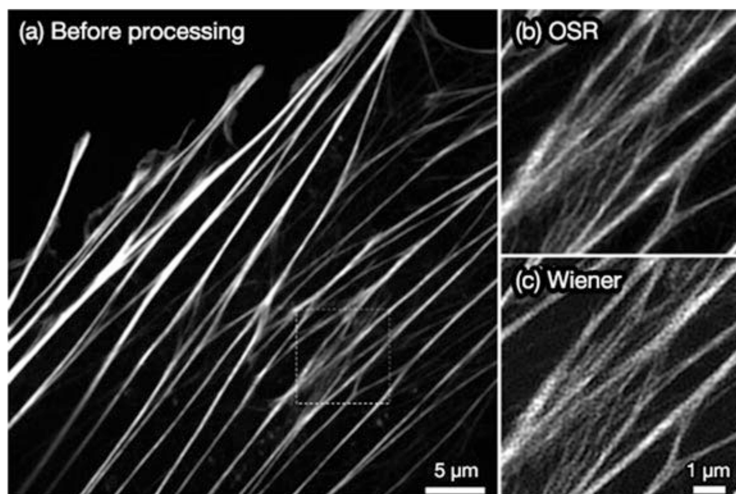


Fig. 11.7 Fluorescence images of stained actin filaments in a fixed cell. (**a**) Confocal fluorescence image acquired by the IXplore SpinSR system. (**b**, **c**) Magnified image of the highlighted portion (**a**) with the OSR filter (**b**), with a Wiener filter (**c**). Image utilized with direct permission of Olympus America [15]

about a specimen in motion or a process which is occurring in real time. Even if a specimen is scanned fairly quickly and multiple times, the data would still reflect large gaps in time. While spinning disk microscopy could be related to watching a person walk across a room, laser scanning confocal microscopy would be similar to watching that same person walk across a room while a strobe light is flashing. There are gaps in the information gathering due to the use of a single pinhole for image acquisition.

That is not to say that spinning disk microscopy does not have drawbacks. While spinning disk microscopy is very fast compared to laser scanning confocal microscopy, its confocality is limited. This means the optical sections tend to be thicker and less optimizable. Spinning disk microscopy is also limited by depth. Due to imaging speed the excitation signal is extremely brief, while providing for the ability to image with lower toxicity it in turn limits the time frame and signal can be evoked and elicited from a sample. A dim quick pulse of light will dissipate more quickly due to scattering; there is simply less incident light so the light will not travel as deep into the sample. The lessened excitation energy also limits the utilization of some applications. Spinning disk microscopy is excellent for visualizing many cellular processes; however, it also lacks the intensity required for purposeful photobleaching or uncaging of advanced fluorophores. A researcher needs to match the data acquisition needs to the type of imaging system which can best provide that data output.

Spinning disk microscopy is a valuable tool for researchers. When in need of an imaging modality with low toxicity, fast acquisition times, and the ability to pick up confocal signals in a live environment then spinning disk microscopy is the best

choice. Geared toward the imaging of cellular processes, protein interactions, vesicular motion, and microorganism interactions, spinning disk microscopy provides for the scientific exploration of how life lives.

11.8 General Questions

1. *What are the advantages of using a spinning disk microscopy system?*
2. *What aspects of the Nipkow disk provide for confocality?*

11.9 Chapter Summary

The need for ultrafast confocal imaging for biomedical research has fueled the optimization of spinning disk microscopy for live-cell applications. Flexibility in light sources and detection systems provides researchers a customizable solution for imaging purposes. The imaging of living samples poses several complications not necessarily seen in fixed tissues or other non-living specimens such as low signal emission, specimens in motion, and the utilized reporters and incident energies being toxic to the living cells which pose the risk of altering the processes being studied. By using spinning disk microscopy, a researcher can modify the system to optimize the experiments and drastically reduce detrimental effects to living samples.

Answers to Questions

1. Spinning disk microscopy offers the advantage of imaging live samples in motion in a biologically friendly manner.
2. The pinholes act as a barrier to out-of-focus light scattered by the sample.

Take-Home Message

- Spinning disk microscopy is used to image living, moving, and/or photo-fragile specimens.
- Spinning disk systems are highly customizable providing a broad spectrum of imaging capabilities.
- Just as the name suggests, all spining disk microscopy systems utilize pinholes in one or more disks which spin through the light path to build an optical image.

Further Reading and Tutorials

- Scientific Volume Imaging (svi.nl/HomePage)
- Molecular Expressions (micro.magnet.fs.edu/micro/about.html)

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