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Imaging intracellular protein dynamics by spinning disk confocal microscopy

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Abstract

The palette of fluorescent proteins has grown exponentially over the last decade, and as a result live imaging of cells expressing fluorescently tagged proteins is becoming more and more main stream. Spinning disk confocal microscopy (SDC) is a high speed optical sectioning technique, and a method of choice to observe and analyze intracellular fluorescent protein dynamics at high spatial and temporal resolution. In an SDC system, a rapidly rotating pinhole disk generates thousands of points of light that scan the specimen simultaneously, which allows direct capture of the confocal image with low noise scientific grade cooled charged-coupled device (CCD) cameras, and can achieve frame rates of up 1000 frames per second. In this chapter we describe important components of a state-of-the-art spinning disk system optimized for live cell microscopy, and provide a rationale for specific design choices. We also give guidelines how other imaging techniques such as total internal reflection (TIRF) microscopy or spatially controlled photoactivation can be coupled with SDC imaging, and provide a short protocol on how to generate cell lines stably expressing fluorescently tagged proteins by lentivirus-mediated transduction.

1. Introduction

The discovery of the original green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, the realization that GFP could be used to label cells and proteins, and subsequent development of an entire color palette of fluorescent proteins (FPs) from different organisms have revolutionized cell biology (Chalfie et al., 1994; Shaner et al., 2005; Shimomura et al., 1962; Tsien, 1998; Zimmer, 2009). Over the last ten years, thousands of papers have been published employing FPs to label cells or intracellular components. Such experiments were substantially more difficult before the advent of GFP and required *in vitro* fluorescent labeling of purified proteins and cumbersome microinjection into tissue culture cells (Sammak and Borisy, 1988; Wittmann et al., 2003; Wittmann et al., 2004). The availability of genetically encoded FP tags has allowed specific labeling of virtually any protein component in living cells and has been a major driving force of discovery in cell biology and innovation in fluorescence light microscopy.

Out-of-focus light obscures detail in conventional, wide-field epifluorescence microscopy, which is particularly problematic with FP-tagged proteins that often have a high cytoplasmic background. Hence, the demand has risen for confocal techniques that allow rapid, multi-dimensional imaging of FP-tagged protein dynamics in live cells. All confocal microscopes

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rely on the same concept that was initially invented by Marvin Minsky in the 1950's. Pinhole apertures placed in the emission light path allow light to pass that originates from the point of focus, but largely block light from out-of-focus planes. Confocal laser scanning (CLS) microscopes utilize a single excitation laser beam that is rapidly scanned over the specimen. The light returning from each point through the pinhole is typically quantified by a photomultiplier tube and the image is reconstructed by a computer (Conchello and Lichtman, 2005). In contrast, spinning disk confocal (SDC) microscopes use a rapidly rotating disk with thousands of pinholes such that thousands of points of light scan the specimen simultaneously (Fig. 1). The spinning disk design is sometimes referred to as Nipkow disk because of its similarity to rotating pinhole disks used for early experimental TV transmissions in the late 1800's. However, spinning disk systems were not used for fluorescence microscopy until recently because it was not possible to transmit enough excitation light through the pinhole disk to the specimen to obtain a sufficiently bright confocal signal. In combination with high intensity laser light sources and sensitive digital cameras, this problem was overcome by introduction of a dual disk design introduced by the Yokogawa Electric Corporation ~10 years ago in which incoming excitation light is focused through the pinholes by a second disk containing microlenses that are aligned with the pinholes on the Nipkow disk (Tanaami et al., 2002). In the Yokogawa design, the pinholes are arranged in a spiral array of equal pitch to generate uniform illumination of the specimen. Longer wavelength emission light returning from the specimen is reflected out of the excitation light path by a dichromatic mirror located between the two disks (Fig. 1).

SDC microscopy has a number of important advantages for fluorescence live cell imaging compared with single point CLS systems. Because the specimen is scanned by thousands of points of light in parallel, the rate of frame acquisition is high. SDC microscopes can achieve theoretical frame rates of up to 2000 frames s^{-1} , and clearly this is not the limiting factor for most cell biology live imaging experiments. Fast frame rates have the advantage that scientific grade cooled charge-coupled device (CCD) cameras can be used for direct confocal image acquisition rather than photomultiplier tubes that are still the light detectors most often employed in CLS microscopes. Although photomultiplier tubes can achieve extremely high gain, they are typically limited by low quantum efficiency and high noise as a result of the charge multiplication process. Thus, in a real life setting, modern cooled CCD cameras will always produce a better, less noisy image and the development of large format, high resolution, and low noise scientific grade complementary metal-oxide-semiconductor (CMOS) cameras holds additional promise for the future.

SDC microscopy is also less prone to fluorescence saturation in which high illumination intensity results in the majority of fluorophores populating the excited state. In this situation, additional excitation light only contributes to photodamage and does not yield additional signal. During a typical camera exposure of a few hundred milliseconds on an SDC system, each point in the specimen is illuminated several hundred times. The image formed results from many short exposures at comparably low peak illumination intensity. In contrast, each point is illuminated only once during the acquisition of one frame on a CLS microscope resulting in longer dwell times per pixel and several thousand fold higher peak illumination intensities. Thus, fluorescence saturation is reached quite easily on a CLS microscope (Wang et al., 2005), and is most likely the reason for anecdotal reports of increased photobleaching compared with SDC microscopy.

One disadvantage of spinning disk and other multi-point confocal systems is that the theoretical confocality is somewhat reduced compared with single point laser scanning systems (Conchello and Lichtman, 1994). The reason for this is that some out-of-focus emission light will be transmitted to the camera through adjacent pinholes. In addition, spinning disk systems have a fixed pinhole size that is optimized for 100 $\times$ magnification. In

theory these pinholes are too large at lower magnification resulting in transmission of more out-of-focus light and reduced confocality. However, in reality this slight loss of confocality is more than compensated by a better signal-to-noise ratio and reduced photobleaching.

Different variations of fast multi-point confocal scanning systems are now available, but the original dual disk design by Yokogawa is still the most successful and most widely used. It is utilized by different companies offering turn-key SDC microscopes including the Andor Revolution and the Perkin-Elmer UltraView systems. The objective of this chapter is to give an overview of a spinning disk confocal microscope system that we have recently built in our lab (Fig. 2). We highlight aspects of instrument design that we think are essential for optimal live cell imaging performance, and provide a rationale for specific design choices. This is important because spinning disk confocal imaging systems always combine components from many different manufacturers, and the combined effect of multiple small design mistakes can add up to overall sub-optimal performance of the system. It is thus essential for anybody who wishes to set up their own SDC microscope to understand and think about what is expected from the instrument and how different components will affect the capabilities of the system.

2. Instrument Design

2.1. Microscope, stage, and environmental control

An SDC microscope does not only consist of the confocal head, but careful consideration should be given to other optical and mechanical components of the system. An inverted microscope stand is necessary for the observation of live specimens in open tissue culture chambers. Mammalian cells will also require temperature and may require CO₂ control. We have found that the best way to provide a stable environment is an acrylic box that encloses the entire microscope stage, and that should be sufficiently large to allow easy manipulation of the specimen. Such incubators are available from different manufacturers. We are using an environmental chamber made by In Vivo Scientific in which warm air enters the incubator from below and is recycled to the heater at the top, a design that minimizes temperature gradients in the incubator chamber. We always leave the temperature control at 37°C, and also store objective lenses and stage adaptors for different cell culture chambers inside the incubator. It takes several hours for the microscope body to reach temperature equilibrium during warm-up, and temperature fluctuations will cause focus drift. In general, focus drift can be a major problem in any high magnification imaging experiment. Particularly if quantitative analysis is to be performed, shifts in focus over time can invalidate otherwise perfectly good datasets. For these reasons, we are using a Nikon TI inverted microscope stand that includes Nikon's proprietary Perfect Focus technology. The Perfect Focus system utilizes a low power infrared laser beam that is partially reflected on the interface between the cover glass and the aqueous media above. The reflected beam is a sensitive readout for the position of this interface and directly controls a focus feedback mechanism. It is important to note that this works well with live specimens in which there is a large difference in refractive index between cover glass and tissue culture media, but often fails with fixed samples in which the refractive index of the mounting media is too similar to that of glass. For imaging with the highest possible optical resolution we typically use Nikon CFI Apo TIRF N.A. 1.49 100× or 60× oil immersion objective lenses. For thicker specimens, we empirically adjust the coverslip thickness correction collar on these lenses to minimize spherical aberration, but we have also used a Nikon CFI Plan Apo N.A. 1.2 60× water immersion lens for 3D specimens. An MS-2000 motorized stage from Applied Scientific Instrumentation with linear encoders is used to be able to record multiple positions during one experiment. At high magnification positional repeatability is extremely important, and in our hands this stage will return to positions within 300 nm accuracy. Positional accuracy can often be improved by reducing stage speed. Nikon NIS Elements

software running on a Windows 7 64-bit PC is used to control all components of the microscope.

2.2. Spinning disk confocal scanner and illumination

We are using both Yokogawa CSU-10 and CSU-X1 spinning disk confocal scan heads on different microscope systems. One advantage of the newer CSU-X1 is a higher and adjustable disk rotation speed, which can be important in combination with short camera exposure times. The pinhole pattern on the Nipkow disk is arranged such that each rotation of the disk scans the image field 12 times. The CSU-10 fixed rotation speed is 1,800 rpm resulting in a rate of 360 scans s^{-1} . Thus, the image formed on the camera chip represents the integration of multiple individual scans by the Nipkow disk, and short exposure times can therefore result in streaking artifacts. For example, in a 5 ms exposure different image areas are scanned either once or twice resulting in a 50% signal variation (Fig. 3B). This can be resolved by synchronization of disk speed and camera exposure time. However, exact timing can be difficult to achieve and an alternative, simpler solution is to increase disk speed so that variations in image coverage do not matter as much (the CSU-X1 can reach up to 10,000 rpm, i.e. 10 scans in 5 ms). In live cell imaging experiments the minimum exposure time is most often dictated by the fluorescence signal that can be obtained from cells expressing FP-tagged proteins at low, physiological levels. We find that high resolution imaging with a non-intensified interline CCD camera (see below) generally requires exposure times of 100 ms or longer at 100% laser power. Thus, streaking artifacts do not present a problem in most experiments.

A weak point in the design of Yokogawa spinning disk heads are the optics by which the excitation laser light is coupled into the spinning disk head. Factory-shipped Yokogawa heads are typically not well aligned, display a relatively large variation of intensity across the field as a result of the Gaussian beam profile exiting the single-mode fiber, and throughput of excitation light to the sample is quite inefficient. For these reasons, we use a CSU-X1 custom-modified by Spectral Applied Research. Although the details of this Borealis modification are proprietary, it involves the use of a multimode optical fiber to couple excitation light into the confocal head, which significantly improves field flatness and increases excitation light throughput several fold over a wide range of laser wavelengths.

The better excitation light efficiency of the Borealis CSU-X1 also allows the use of less powerful lasers. The described system employs four 100 mW diode-pumped solid-state (DPSS) lasers at 442 nm (Power Technology Inc.), 488 nm (Coherent Sapphire), 515 nm (Cobolt Fandango), and 561 nm (Cobolt Jive), in contrast to 200 mW DPSS lasers we use on an older CSU-10 system. 100 mW are significant laser power levels that can cause retina damage, and appropriate laser safety precautions and interlocks should be used. Solid state lasers now offer a large selection of available wavelengths at these power levels, and have a longer lifetime, lower power consumption, develop less heat and are significantly smaller than high power Krypton-Argon gas lasers (Adams et al., 2003). These lasers are mounted in an LMM5 laser merge module by Spectral Applied Research and wavelength and intensity are controlled by an Acousto-Optical Tunable Filter (AOTF). Laser combiners are available from other companies, but in our hands the LMM5 has performed exceptionally well, and on our older setup has not required any alignment in over 4 years. Although the LMM5 is currently not directly supported by NIS Elements software it is easy to control by serial commands and we implemented laser line and intensity selection as general filter changers in the software.

Because laser wavelengths are selected by the AOTF, excitation filters in the spinning disk head are not necessary and should be omitted. We use three dichroic mirrors from Semrock

that we find optimal for imaging GFP alone, or different combinations of FPs (Table 1). Spinning disk dichroic mirrors only transmit narrow bands of excitation light, which is different from dichroic mirrors in epifluorescence filter sets. The dichroic mirrors are mounted in a manual filter changer in our Borealis-modified CSU-X1 because the motorized dichroic mirror changer offered by Yokogawa is expensive and not fast enough for switching during a live cell experiment.

Photobleaching is the number one enemy of FP live cell imaging, and selection of optimized emission filters is extremely important for maximal signal, a fact that is often strangely overlooked in the design of a live cell imaging system. Emission filters are mounted in a fast Sutter Instrument filter changer between the spinning disk head and the camera. For shortest switching time of ~40 ms, combinations of filters that are used together in dual wavelengths experiments should be mounted in adjacent positions. In any case, emission filter switching will result in a delay between image acquisitions at different wavelengths. If speed is of the essence a multiband emission filter can be used, but one should be aware that longer wavelength FPs (e.g. RFP) will be excited by the shorter excitation wavelength (e.g. 488 nm for GFP), and will contribute to signal cross-talk into that channel. To optimize fluorescence signal, we use emission filters with cut-off wavelengths as close as possible to excitation laser lines (Table 1). Bandpass filters should be selected with a wide transmission band, and we use longpass emission filters for the longest wavelength in an experiment to collect as much emission light as possible. For example, imaging of EGFP alone with the GFP/RFP dual wavelengths dichroic mirror and emission filter results in a ~20% loss of signal compared with the optimized GFP longpass set. Thus, acquiring images with similar signal will require more excitation light and result in increased photobleaching.

To further minimize specimen exposure to excitation light, and thus photobleaching, laser shutters should not be controlled through software, but instead be triggered directly by the camera. Most cameras output a transistor-transistor logic (TTL) signal when an image is acquired, which can be used to directly trigger a shutter. However, in multi-wavelength experiments, a simple logic circuit is necessary so that the computer can control which shutter is going to be opened (Fig. 4A). In many systems the software-induced delay in opening and closing shutters can be several hundred milliseconds, and thus significantly contribute to excessive photobleaching. In the described that is controlled by a fast 64-bit PC, and in which camera control during time-lapse experiments takes advantage of enhanced communication between Andor cameras and NIS Elements software, the software-induced delay between shutter opening and camera exposure is much shorter. Nevertheless, at short exposure times, decreased photobleaching is still evident for both EGFP and mCherry in camera-triggered acquisition mode (Fig. 4B).

2.4. Image acquisition

Even in the Yokogawa dual disk design, transmission of excitation light to the sample is inefficient. Spinning disk systems were and are still sold with insufficient excitation laser power, and low-light electron multiplication CCD (EMCCD) cameras are used to compensate for low signal. In any epifluorescence microscope, confocal or not, the objective lens functions both as objective and condenser, and the smallest resolvable distance at which the maximum of one point spread function (PSF) coincides with the first minimum of an adjacent PSF is calculated by the Rayleigh criterion $r = 0.61\lambda/\text{N.A.}$ At 510 nm, the emission maximum of EGFP, and using a 1.49 N.A. objective lens, r is approximately 210 nm. The Nyquist sampling criterion, which states that the sampling frequency must be 2 times larger than the highest frequency of a signal, is often inaccurately referred to in this context. However, in most cases depending on where the center of the circular PSF is projected onto the square pixel grid of the camera CCD, Nyquist sampling is not sufficient to resolve two PSFs separated by r in the digital image (Wittmann et al., 2004). Camera noise will make

this situation even worse. To accurately reproduce the circular PSF image on a square pixel grid without excessive aliasing or signal clipping, the pixel size should be at least 3 times smaller than r ; i.e. ~ 70 nm in the example above.

A typical, high-end EMCCD chip such as the one used in the Andor iXon camera has $16 \times 16 \mu\text{m}$ pixels. At $100\times$ magnification, this translates into a 160 nm pixel size in the image formed on the camera sensor. Thus, although the high sensitivity of EMCCD cameras has the advantage of potentially very low photobleaching, because of the large pixel size significant optical resolution is lost (Fig. 5). In contrast, most high-resolution cooled CCD cameras utilize a Sony Interline CCD chip with $6.45 \times 6.45 \mu\text{m}$ pixels, which in theory yields 64.5 nm pixels on the image. It is worth noting that some intermediate magnification occurs in the spinning disk head, and the measured pixel size in the described system is ~ 60 nm at $100\times$ magnification. The Sony Interline ICX285 chip has been around for over a decade, and is used in cameras from many manufacturers including the Hamatsu ORCA series, Photometrics HQ2, and Andor Clara. Cooling of the CCD chip is an important consideration to minimize dark current, and in direct comparison, the Andor Clara appears to achieve the lowest read noise.

The SDC image is formed in the plane of the Nipkow disk, and it is thus essential that the camera is well focused on the pinholes in the disk. This is done by switching off the spinning disk motor and imaging the pinholes with diascopic illumination from above. The optimal focus is determined by adjusting the distance between camera and the spinning disk head. Pinholes in the Yokogawa disk have a diameter of $50 \mu\text{m}$ and at $100\times$ magnification should appear in the image as evenly illuminated disks with well-defined edges of ~ 500 nm in diameter (Fig. 6). This is slightly larger than the theoretical 420 nm diameter of the Airy disk for a 100×1.49 N.A. objective calculated above.

3. Combination with other imaging techniques

Because both illumination and imaging in an SDC system occur through the camera port, the widefield epifluorescence port remains available for other imaging modalities. In this section, we describe briefly how total internal reflection microscopy (TIRF) or photoactivation techniques can be integrated with a SDC microscope.

3.1. SDC and TIRF

In TIRF the evanescent wave generated by a laser beam that is reflected at the interface between the coverglass and the aqueous medium above only excites fluorophores in very close proximity (100–500 nm) to the cover glass (Mattheyses et al., 2010). Thus, TIRF is particularly useful to observe events at the bottom of the cell with high contrast such as vesicle fusion with the plasma membrane or adhesion site dynamics (Hu et al., 2007; Toomre et al., 2000). In the described system, we are using a motorized Nikon TIRF illuminator in which the incident angle of the TIRF laser beam can be controlled by software. The TIRF image is captured by an iXon EMCCD camera on the right side camera port. The LMM5 laser merge module is outfitted with a software-controlled fiber switch that allows the usage of the same lasers for SDC imaging and TIRF illumination and rapid switching between the two modes. Switching between SDC and TIRF, however, also requires moving the epifluorescence filter turret in the microscope stand between an empty position for SDC imaging and an optimized TIRF filter set (Chroma Technology Corp Cat No. 91032), as well as switching between the left and the right camera port. It makes economic sense to integrate a TIRF illuminator on an SDC system because lasers are expensive. However, long switching times between the different modes on the microscope stand limit its usefulness for simultaneous confocal and TIRF imaging. Although switching between filter cube positions

in the Nikon TI microscope stand is quite rapid, changing from the left to the right camera port is slow and takes about a second.

3.2. SDC and fast photoactivation

Photobleaching, photoactivation or photoconversion of FPs are useful techniques to quantitatively investigate protein and organelle dynamics in live cells (Lippincott-Schwartz and Patterson, 2008; Lukyanov et al., 2005; Shaner et al., 2007). SDC microscopy is particularly amenable to fast photoconversion of FPs because 405 nm light required for photoconversion can be coupled into the widefield epifluorescence port with an appropriate dichroic mirror installed in one of the epifluorescence filter cubes (e.g. Semrock Di01-R405-25×36; Fig. 1). Longer wavelength light used for SDC imaging is transmitted through the dichroic mirror while the shorter wavelength photoactivation light entering through the epifluorescence port is reflected toward the specimen. Near complete photoconversion of mEos2 for example can be readily achieved in this setup with relatively low intensity of photoactivation light (Fig. 7) (McKinney et al., 2009). This setup has the important advantage that SDC imaging and photoactivation can be performed simultaneously. In CLS microscopes, fluorescence recovery after photobleaching (FRAP) experiments are often performed by photobleaching an area of interest with the scanning laser set to maximum intensity, but the delay between photobleaching and acquisition scanning is a severe limitation of this method. This is particularly important for the measurement of rapid diffusion-limited protein dynamics where recovery may already occur during the bleaching process. Such fast dynamics occur for example in proteins binding reversibly to cytoskeleton structures (Wittmann and Waterman-Storer, 2005). One limitation of utilizing 405 nm wavelength light for photobleaching is, however, that many FPs have been optimized to abolish the excitation peak in the near UV, and EGFP for example is bleached rather inefficiently at 405 nm. Photobleaching of EGFP may be more easily achieved with longer wavelength lasers (e.g. 442 nm or even 470 nm) that could still be coupled into the SDC light path as described.

4. Specimen preparation

In order to image intracellular FP dynamics, the cells that are to be imaged have to express the FP-tagged protein of interest. Transient expression can be achieved by a variety of different commercially available transfection reagents. However, many cell types are not easily transfected, and most, if not all, transfection reagents are cytotoxic and may adversely affect cell behavior. Some of these problems can be overcome by infection with recombinant adenovirus particles that yield high infection efficiency with most cell types (Gierke et al., 2010). However, protein expression levels in transiently expressing cells always suffer from high cell-to-cell variability and generally excessively high expression levels. Clearly, the gold standard is to generate stable cell lines that express FP-tagged proteins at low and relatively even levels across the population. Although this can be achieved by transfection, antibiotic selection and subsequent fluorescence-activated cell sorting (FACS), we find that lentivirus-mediated transduction is the most reliable technology to rapidly generate stably expressing cell lines (Vigna and Naldini, 2000). Lentiviral transduction has many benefits over both transfection and adenovirus transduction. Lentivirus will stably integrate into the host cell genome resulting in more physiological expression level. This is extremely important in quantitative live cell imaging experiments, as overexpression of FP-tagged proteins will alter the dynamics of the process under investigation.

4.1. Lentivirus-mediated stable expression

Although reagents and protocols on how to generate stable cell lines by lentivirus transduction are available from many sources, we include a short detailed protocol that has worked well for us to generate many different cell lines stably expressing FP-tagged proteins. Different lentivirus systems use different packaging vectors that are not interchangeable, and we routinely use the ViraPower Lentiviral Expression System (Invitrogen) that is not inexpensive, but has proven to be highly reliable in our hands. It is important to ensure proper biosafety procedures and clearance to work with amphotrophic lentivirus. Although 3rd generation lentivirus systems such as ViraPower produce replication-incompetent lentivirus particles, and have enhanced biosafety features, all local safety guidelines should be followed and American laboratories require BSL-2 clearance. Contaminated surfaces, containers and pipette tips should be decontaminated with 10% bleach before disposal.

A. Required Materials

Gateway entry vector (e.g. pENTR/D-TOPO, Invitrogen Cat. No. K2400-20)
 Gateway destination vector (e.g. pLenti6/V5 DEST, Invitrogen Cat. No. V496-10)
 Gateway LR Clonase II Enzyme Mix (Invitrogen Cat. No. 11791-020)
 One Shot Stbl3 *E. coli* (Invitrogen Cat. No. C7373-03)
 S.O.C medium (Invitrogen Cat. No. 15544-034)
 Lipofectamine 2000 Transfection Reagent (Invitrogen Cat. No. 11668-027)
 ViraPower Lentiviral Packaging Mix (Invitrogen Cat. No. K4975-00)
 Opti-MEM I Reduced Serum Medium (Invitrogen Cat. No. 31985-070)
 293FT Cell Line (Invitrogen Cat. No. R700-07)
 Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Cat. No. 10313)
 supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen Cat. No. 26140), 2mM L-glutamine (Invitrogen Cat. No. 25030), 0.1 mM MEM non-essential amino acids (Invitrogen Cat. No. 11140-050); 500 µg/ml Geneticin (Invitrogen Cat. No. 10131-035) is added for 293FT maintenance, but not during lentivirus production.
 Sequabrene (Sigma, Cat. No. S-2667)
 Blasticidin (Invitrogen R210-01)

B. Generation of lentiviral destination vector—We routinely use Gateway cloning technology (Invitrogen) to create entry and destination clones for lentivirus production. The first step is the generation of an entry clone with your gene of interest. The exact cloning strategy will depend on the desired product, but we usually include the FP tag at this point as well as additional unique restriction sites in case we want to use the DNA construct for traditional cloning into another vector at a later date. A Kozak consensus sequence and appropriate stop codons should be included in the entry clone. Gateway cloning into the destination vector is essentially done following Invitrogen protocols with reduced reaction volumes to conserve reagents¹. Briefly, mix 25 ng of the entry clone and 50 ng of pLenti6/V5 DEST in 4ul water, add 1 µl LR Clonase II Enzyme Mix, mix by pipetting up and down. Incubate for at least 1 hour at room temperature. We generally incubate overnight for inserts longer than 5 kb. Stop reaction by adding 0.5 µl Proteinase K solution and incubate at 37°C

¹Useful website for frugal Gateway Cloning Protocols: <http://www.untergasser.de/lab/protocols/index.htm>

for 10 minutes. Transform the entire reaction using Stbl3 competent cells following Invitrogen's instructions. Stbl3 cells are recommended as they reduce the frequency of unwanted homologous recombination of lentiviral long terminal repeats (LTRs). The LR reaction is very efficient and to ensure single colonies, plate several dilutions on ampicillin plates. We generally isolate plasmid DNA from 5–10 clones to screen by sequencing. Ensure that the mini-prep protocol uses a resuspension buffer containing 10 mM EDTA required to inactivate the Stbl3 endonuclease. Contamination-free DNA used for 293FT transfection should be prepared with a commercial Midi- or Maxi prep kit.

C. Lentivirus production—293FT cells produce the highest quality lentivirus when they are carefully handled and maintained. Unlike adenovirus, lentivirus is secreted into the medium and cell health is essential. It is important to ensure that the 293FT cells never overgrow, and are passaged at least twice before lentivirus production. We do not use cells after passage 10. Take care when changing growth medium as 293FT cells become easily detached. When changing media, we recommend removing the old growth medium by decanting, and slowly adding fresh DMEM to the edge of the dish. Cells will detach from the dish if left without medium even for short periods of time, and if multiple transfections are carried out in parallel, growth medium changes should be carried out one dish at a time.

Day 1: Split 293FT cells into complete, but Geneticin-free DMEM growth medium, following Invitrogen's instructions. Cells should be 80–90% confluent the following day, and we generally plate 2–3 different dilutions in 6 cm tissue culture dishes to ensure cells are at the optimal confluency.

Day 2: Co-transfect 293FT cells with the ViraPower packaging mix and the destination clone according to Invitrogen's instructions. We commonly use Lipofectamine 2000. Other transfection reagents can be used but will require optimization. We divide all reagent quantities by 2.8 to account for the smaller surface area of 6-cm dishes. It is important that the transfection is performed in the absence of Geneticin. Add transfection mixture dropwise and very gently mix by rocking plate front to back, then side to side. Do not swirl.

Day 3: Carefully replace growth medium with fresh DMEM without Geneticin. Remember that you are now handling active, infectious virus particles. Do not be too concerned if cells at the edge of the dish become detached slightly. Expression of the VSV-G glycoprotein causes 293FT cells to fuse resulting in large, multinucleated cells. This morphological change is normal and does not affect lentivirus production.

Day 4: Harvest lentivirus by pipetting cell supernatants into 15 ml conical centrifuge tubes. We use serological pipettes or filter tips when handling virus. If the 293FT cells remain attached to the tissue culture dishes during this process, gently add fresh DMEM for a second round of virus harvest the following day. We have found no difference in virus harvested at 48 or 72 hours post-transfection. Centrifuge supernatants at 3000 rpm for 15 minutes at 4°C to pellet cellular debris. Snap freeze lentivirus-containing supernatant in 1ml aliquots in cryovials in liquid nitrogen and store at –80°C. Lentivirus particles can be further concentrated and purified using the PEG-it virus precipitation system (Systems Biosciences), or filtration through 0.45 µm syringe filters, although this is not necessary for production of simple stable cell lines (Reiser, 2000).

D. Production of stable cell line—Split cells to be 70–80% confluent at the time of transduction. We generally use one 6 cm dish for each cell line we want to create. To each dish, add 1 ml of growth medium, 0.1–1 ml of virus and 1–2 µg/ml Sequabrene, a cationic polymer that neutralizes the charge between virions and sialic acid on the cell surface and

greatly increases infection efficiency (Davis et al., 2004). The exact amount of virus will depend both on virus titer (Sastry et al., 2002) and on the cell type used, but 1 ml should generally be more than enough. Rock dish side to side and back and forth, return to tissue culture incubator overnight. Change growth medium the next day remembering that the old medium still contains active virus. After 24 hours start Blasticidin selection, making sure that cells will have enough room to divide. The exact concentration of Blasticidin (between 2–10 µg/ml) will need to be determined for the cell type used. We always include a control dish to ensure that Blasticidin is efficiently killing non-transduced cells. Change the growth medium every 2–3 days to remove dead cells. We generally collect approximately 6 million cells by FACS sorting, selecting cells in the top 20% of fluorescence intensity.

4.2 Epithelial sheet migration assay

Cell migration is often analyzed by wounding of a cell monolayer. Traditional methods to generate such wounds in confluent fibroblast cultures involve scratching of the cell monolayer with a pipette tip, and subsequent imaging of the closure of this “wound”. For a number of reasons, however, we find that epithelial cells are better suited to investigate cytoskeleton dynamics during cell migration. Epithelial cells that retain cell-cell contacts, for example HaCaT keratinocytes, migrate as a cell sheet, and leading edge cells become highly polarized for long periods of time resulting in highly coordinated turnover of cytoskeleton structures (Kumar et al., 2009). However, scratching of an epithelial cell monolayer with a pipette tip usually results in excessive tearing of the wound edge, detachment of leading edge cells from the cover glass, and disturbance of the deposited extracellular matrix. This disrupts coordinated cell migration and is particularly problematic for high resolution SDC imaging. We therefore use a modified scratch assay in which half of the epithelial monolayer is removed from the cover glass by a razor blade swipe.

A. Required Materials

15 mm round #1.5 coverslips (Warner Instruments CS-15R15, Cat. No. 64-0713). To clean coverslips for live cell imaging, immerse coverslips in a glass jar in deionised water with 1–2 drops of Versa-Clean detergent (Fischer Scientific, Cat. No. 04-343), and sonicate for 20 minutes. Rinse 5–6 times in deionised water until detergent bubbles disappear, sonicate for 20 minutes, rinse again 5–6 times in water, and finally rinse 2–3 times in ethanol. Store the coverslips in ethanol. Flame coverslips briefly to remove ethanol before placing in tissue culture dishes.

Custom-made anodized aluminum imaging chambers; these are slide size pieces of aluminum with a central hole and a shallow counterbore on either side to hold coverslips (Wittmann et al., 2004).

High quality stainless steel razor blades (e.g. GEM brand, single edge industrial blade 62–0165) Appropriate cell culture growth medium.

0.25% Trypsin-EDTA (Invitrogen Cat. No. 25200-056)

1 M HEPES buffer solution (Invitrogen Cat. No. 15630-080)

High vacuum grease (Dow Corning Cat. No. 1597418)

B. Epithelial migration assay

Day 1: We generally plate cells 2 days prior to wounding at several different dilutions to ensure ~90% confluency on the day of wounding. This allows epithelial cells to secrete extracellular matrix, and to properly adhere and spread. Coating coverslips may also improve migration, and for HaCaT keratinocyte migration assays we usually coat coverslips

with 10 $\mu\text{g/ml}$ Fibronectin (Roche Cat. No. 11051407001) in PBS for 30–40 minutes at 37°C, 5% CO_2 . Rinse coated coverslips with PBS before plating.

Day 3: Perform the wound assay in the morning following sterile tissue culture practice. Wash coverslips in 37°C PBS and return to incubator for 5 minutes. This is important and allows cell-cell contacts to loosen, and prevents tearing of the cell sheet during wounding. Place coverslip on parafilm with cells facing up. Place the edge of a sterile, sharp razor blade in the middle of the coverslip, and while holding the coverslip on one side, scrape the blade across half of the coverslip to remove all cells from this half. Take care not to apply too much force, or the coverslip will break. Return coverslip to a tissue culture dish, and immediately cover with 0.25% Trypsin-EDTA for 10 seconds, then rinse 5–6 times by directly pipetting media across the wound to remove loosely attached and dead cells. Return to incubator for 2–3 hours.

Prepare live cell imaging chamber by coating both sides of the rim with a thin layer of vacuum grease. This works best by squeezing grease out of a plastic syringe. Remove excess grease from the inside of the rim. Place a clean coverslip onto the greased rim, gently pushing down on the coverslip edge. Clean with ethanol to remove grease on the coverslip. Flip chamber over, so that the coverslip-mounted side is facing down. Add 150 μl of media, supplemented with 20 mM HEPES pH 7.5 to the chamber well. Mount coverslip with cells onto the greased rim, gently pressing down and aligning the wound to ensure it is vertical when on the microscope. Do not push on the center of the coverslip, as this will create air bubbles or break the coverslip. Clean the outside coverslip surface with ethanol. Make sure it 100% clean as this will face the objective. Place in tissue culture incubator overnight and image the following day.

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Figure 1. Diagram of SDC microscope light path, also indicating the position of an optional dichroic mirror for coupling of a photoactivation light source.

Figure 2.

(A) Photograph of the described SDC microscope system. (a) In Vivo Scientific environmental chamber, (a1) CO₂ supply for environmental control; (b) Nikon TI inverted microscope stand; (c1) Applied Scientific Instrumentation MS-2000 linear encoded stage, (c2) joystick, and (c3) rack-mounted controller; (d) rack-mounted 64-bit Windows 7 PC; (e) Yokogawa CSU-X1 spinning disk confocal head; (f1) Spectral Applied Research LMM5 laser merge module, (f2) LMM5 controller, and (f3) individual DPSS laser control units; (g) Sutter Instrument emission filter changer; (h) Andor Clara Interline CCD camera; (i) Andor iXon EMCCD camera for TIRF; (j) Sutter Instrument Lambda XL Xenon lamp for epifluorescence illumination; (k) camera port for widefield epifluorescence or diascopic imaging. (B) Close-up of Borealis-modified CSU-X1 SDC scan head. (e1) manual dichroic mirror changer, (e2) multimode optical fiber (from LMM5), (e3) connector for disk rotation speed control (from LMM5 controller), (e4) built-in laser safety shutter button (cannot be software-controlled); (g) Sutter Instrument emission filter changer; (h) Andor Clara Interline

CCD camera; (h1) focus tube with lock screws to adjust distance between camera and Nipkow disk; (h2) TTL-out connector (“fire” from Andor cameras to AOTF controller); (i1) Nikon TIRF attachment is visible behind the environmental chamber, (i2) optical fiber to TIRF attachment (from LMM5). (C) Measured XZ point spread functions in widefield epifluorescence (through port k), and SDC mode with 100× 1.49 N.A. objective lens. (B) Comparison of point spread function in widefield and spinning disk confocal microscopy.

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Figure 3.

Streaking artifacts resulting from unequal coverage of the specimen at low disk rotation speeds and short camera exposure times. The streaking pattern becomes negligible if disk rotation speed is increased. The specimen shown are HeLa cells stably expressing mCherry-histone acquired with a 100× 1.49 N.A. objective lens. Scale bars, 10 μm .

Figure 4.

Shutter control by camera trigger decreases photobleaching. (A) Diagram of a simple TTL circuit to switch between two excitation laser wavelengths and trigger shutter opening through the camera. The laser wavelength can be selected by the microscope control software through a pin on the parallel port or better through an inexpensive USB DAQ-board (e.g. from National Instruments). For more than two wavelengths more extensive TTL circuits can be designed. (B) Photobleaching curves of cells expressing EGFP- or mCherry-tagged proteins acquired at 50 ms exposure times and 100% laser power. Particularly the less photostable mCherry bleaches significantly faster when the laser shutter is controlled by the imaging software and not directly triggered by the camera.

Figure 5.

Comparison of images acquired by epifluorescence widefield and SDC microscopy acquired with either a Clara interline CCD camera (Andor) or an iXon EMCCD camera (Andor). Images are displayed at the same magnification, and insets show the indicated regions at higher magnification demonstrating aliasing with the large pixel EMCCD camera. The specimen shown are HaCaT keratinocytes stably expressing EB1-EGFP acquired with a 100× 1.49 N.A. objective lens. Scale bar, 10 μm .

Figure 6.

Wrong focal distance between camera CCD and Nipkow disk results in severe image deterioration. The specimen shown is an EGFP-tubulin expressing cell acquired with a 100 $\times$ 1.49 N.A. objective lens. The camera was moved by the indicated distance away from the optimal position at which pinholes are focused on the camera chip (left panel), and appear as evenly illuminated disks with well defined edges. Insets show images of the stopped Nipkow disk in transmitted light illumination.

Figure 7.

Photoconversion of mitochondria matrix-targeted mEosS2 in mouse fibroblasts imaged by SDC illustrating mixing of mitochondria content. Photoconversion was performed with a Mosaic Digital Illumination device (Andor) and a 405 nm DPSS laser coupled into the SDC light path as described. Scale bar, 10 μ m

Table 1

Dichroic mirrors and emission filters used in the light path of the described spinning disk confocal system. FPs listed are examples and many other FPs will fall into these bands. Particularly red FPs vary widely in their excitation and emission characteristics and 561 nm excitation is a compromise.

Spinning disk head dichroic mirrors		
	Semrock part number	> 90% Transmission
GFP; EGFP	Di01-T488	405–488 nm
	Di01-T405/488/561	400 – 410 nm
GFP; EGFP		488 nm
RFP; tdTomato; mApple; mCherry		561 nm
ECFP; Cerulean	Di01-T445/515/561	441 – 449 nm
EYFP; Venus		513 – 517 nm
RFP; tdTomato; mApple; mCherry		559 – 563 nm
Emission filters		
	Semrock part number	> 93% Transmission
GFP; EGFP (Long-pass)	BLP01-488R	505 nm –
GFP; EGFP (Band-pass)	FF02-525/50	500 nm – 550 nm
RFP; mCherry; etc (Long-pass)	LP02-561RS	569 nm –
ECFP; Cerulean (Band-pass)	FF02-475/50	429 nm – 522 nm
EYFP; Venus (Long-pass)	BLP01-514R	529 nm –