1 In biological imaging, confocal laser scanning microscopy (CLSM) has in the last decade
2 significantly extended our ability to visualize highly complex samples as multidimensional
3 datasets (space, time, colors). In parallel, the introduction of fluorescent protein variants as
4 in vivo tags of structures of interest has opened up new ways to observe cellular processes
5 inside the living cell or tissue

6 The most common type of confocal microscope uses a single focused laser beam to
7 sequentially point-scan a region (single beam confocal microscope, SBCM). The
8 fluorescence created by the passage of this focused beam through the sample is sent
9 through a narrow aperture in the intermediate image plane (the confocal pinhole) onto a
10 detector and is thus reduced to the photons coming from the plane of focus of the objective,
11 but not from regions above or below it. By this rejection of out-of-focus contributions an
12 optical section is created containing only the information from the focal plane. This basic
13 operational principle as it was already realized for Marvin Minsky’s prototype in 1955 is
14 used in most commercially available confocal microscopes today.

15 Long before confocal microscopes became a standard imaging tool in biology, however,
16 another more parallelized approach to confocal imaging was developed using a technique
17 significantly predating most electronic imaging inventions: In 1884 Paul Nipkow created a
18 device that transmitted images electrically. It was the first television camera and made use
19 of a rotating disk with a spiral pattern of holes that broke down two-dimensional
20 information into a sequential series of signals that could be reconstituted into an image
21 using a complementary disk with the same pattern.

22 In 1968, M. Petrán and his collaborators applied the Nipkow disk principle to develop a
23 tandem scanning reflected light microscope in which the single beam scanning confocal
24 approach was parallelized to utilize multiple beams and corresponding pinholes. Although
25 this approach overcame the severe speed disadvantage of the single beam scanning method,
26 it had significant problems of its own. For fluorescence imaging, the technology suffered
27 from little excitation light reaching the sample due to the limited pinhole area
28 (approximately 1%). Additional drawbacks were the requirement of high precision in the

pinhole placement for designs with opposing excitation and emission pinholes or problems with the scattered excitation light inside the detection system for setups using the same pinhole for excitation and emission.

When confocal microscopes became more widely available due to improvements in computer and imaging technology, the favored approach was single beam scanning. Recently, a significantly improved disk design by Yokogawa Inc. as well as progressive improvements in camera design have re-established the alternative multi-beam scanning technique (multi-beam confocal microscope, MBCM), especially for the requirements of in-vivo imaging. In combination with the Green Fluorescent Protein (GFP) technology applied in live-cell imaging, fast, multi-beam scanning microscopy is now a powerful tool for cell biology.

The principle of operation of spinning disk microscopes will mainly be explained referring to a confocal scanning unit (CSU) designed by Yokogawa Corp. It represents a very modern variant of the basic concept and in its design some of the initial limitations of the tandem scanning technology (little excitation light, uneven illumination, backscattering) were addressed and overcome. It is incorporated into several commercially available spinning disk setups (Perkin Elmer, VisiTeC).

A spinning disk confocal microscope consists of a rotating disk with multiple pinholes and a CCD camera. The pinholes on the disk are arranged in a pattern that allows every location of an image to be covered when the disk is rotated.

In spinning disk microscopes an even field of illumination is created (e.g. by widening laser illumination into a circular field) that irradiates a section of the spinning disk. While most light does not pass the disk, the light going through the pinholes forms a set of minibeams corresponding to the pinhole pattern and sweeps the image field because of the disk rotation. Every mini- beam in itself is confocal, with the same aperture serving as the excitation as well as the emission pinhole for a single mini-beam. Designs using opposing pinholes on the disk are not realized in the current instruments.

56 To avoid crosstalk between the spots of individual minibeams, the pinholes are spaced
57 significantly apart. Accordingly, only a small area of the disk is covered by pinholes (1–
58 4%) and most of the excitation light does not reach the sample because it is blocked by the
59 disk. In the Yokogawa design, this problem is overcome by a second disk in front of the
60 pinhole disk. It contains microlenses arranged in the same pattern as the pinholes. These
61 collect the excitation light and focus it into the pinholes thereby significantly increasing the
62 excitation light throughput from approx. 1% to 40–60%.

63 The characteristic differences between SBCMs and MBCMs consist in (1) serial against a
64 parallelized scanning approach and (2) the mode of detection (PMTs vs CCD camera). All
65 further differences between SBCMs and MBCMs result from these two initial factors. In a
66 SBCM the image acquisition rate is limited by the speed of the scan mirrors. The fastest
67 scanning units currently available are operating at resonance frequency, thereby achieving
68 512-line-frame rates close to video rate or more than 100 Hz for reduced 32-line frame
69 formats. In a MBCM the image acquisition rate is limited by the speed of the camera
70 readout. In theCSU-10 version of the Yokogawa scan head the rotation frequency of the
71 disc was limiting the acquisition rate to 360 Hz, which is no longer the case for the CSU-21
72 that can rotate with higher speeds.

73 In single beam scanning, a high intensity laser beam passes over the sample and illuminates
74 every region intensely, but only for a very short period of time (typically 2–3 µs). Only the
75 light put into the sample and read out from the sample during this time is available to
76 transport the information for the generation of the image. As a result, the excitation light
77 has to be intense in order to excite enough fluorophores during this short time. In multi-
78 beam scanning, the excitation light is split into many mini-beams of correspondingly lower
79 intensity. However, several of these beams pass over the same region sequentially and the
80 emission of all their passes is collected during the exposure time of the camera expo- sure.
81 The illumination time per pixel is therefore significantly (1000-fold) longer.

82 Fluorescence Recovery after Photobleaching (FRAP) or photoconversion of fluorescent
83 proteins are powerful techniques to investigate protein dynamics inside living cells. These

84 techniques require a bleaching or activation step, i.e. a short irradiation of a defined image
85 sub-region with intense laser light and is easily performed on SBCMs. Although some
86 spinning disk systems also use laser light for excitation, the laser cannot be used for spot or
87 region bleaching in the existing setups. It is widened for the illumination of the whole field
88 of view and cannot be specifically positioned within the image. Regional bleaching on
89 spinning disk systems could however be performed with an additional positionable laser.
90 This would require customization of the system, but it would be a formidable application
91 considering the excellent fast time- lapse capacity of spinning disk systems.