Methods in Molecular Biology 2779

Springer Protocols

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Flow Cytometry Protocols

Fifth Edition

MOREMEDIA

Humana Press

Chapter 2

Basic Principles of Flow Cytometer Operation: The Make your Own Flow Cytometer

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Abstract

Flow cytometry is a critical technology for biomedical analysis and is an essential component of almost any study of the immune system. Widespread usage and increasing instrument complexity have, however, led to increasing neglect of education in their basic operating principles, a common situation with many technologies. This chapter describes the basics of flow cytometer operation using the Make Your Own Flow Cytometer (https://www.cytometryworks.com), a working cytometer than can be assembled by students into a functional instrument. This project and others like it is seeing widespread usage in biomedical education and can serve as models for like-minded investigators who wish to build their own systems. They also provide a good mechanism to introduce the key operational principles of flow cytometry as illustrated here.

Key words Flow cytometry, Laser, Hydrodynamic focusing, Dichroic mirror, Bandpass filter, Photomultipler tube, Photodiode, Preamplifier, Amplifier, Digitizer, Analog-to-digital converter, Digital signal processor

1 Introduction

Flow cytometry is an indispensable technology in cell biology and biomedical research, particularly in the analysis of the immune system [1]. Flow cytometers allow the analysis of individual cells in a hydrodynamically focused liquid stream, using lasers and sensitive detectors for light scattering measurement of cell size and optical density and fluorescence detection of cellular proteins, intracellular structures, and physiological status [2]. Modern fluorescence-based flow cytometers can detect more than 40 fluorescent markers simultaneously, thus allowing precision analysis of the complex cell mixtures that make up the immune system [3, 4]. The high-throughput nature of flow cytometry allows millions of individual cells to be rapidly analyzed, permitting the

Teresa S. Hawley and Robert G. Hawley (eds.), *Flow Cytometry Protocols*, Methods in Molecular Biology, vol. 2779,
https://doi.org/10.1007/978-1-0716-3738-8_2,

detection of rare cell populations and identification of new cell subsets. Cell sorters, flow cytometers equipped for physical cell separation, permit isolation of individual cell populations for further analysis.

While the technology of flow cytometry has advanced considerably since its inception nearly 50 years ago, the basic optical and fluidic principles behind instrument operation have not changed. Like many advanced technologies, flow cytometers have largely evolved into "black boxes" where students new to the field are unaware of the many basic physical principles behind their operation. In the spirit of many similar programs in the technology "maker" culture, we have designed the Make Your Own Flow Cytometer system, a fully functional instrument that can be assembled from individual parts into a working instrument. This system is based on the original Build Your Own Flow Cytometer system developed by engineers and educators at the National Flow Cytometry Resource (NFCR) located at the Los Alamos National Laboratories (LANL) in the 1990s, one of the birthplaces of the modern cell sorter. The original Build Your Own system continues to see wide usage in flow cytometry education programs. The newer Make Your Own Cytometer is smaller and lighter, allowing it to be transported to flow cytometry educational workshops around the globe. As of 2023, it has been used as an educational tool in flow cytometry workshops held in over 18 countries in all 6 inhabited continents. It heavily relies on open-source, three-dimensional (3D) printed components, giving scientists and educators the inspiration and opportunity to construct their own system. A fully assembled system is shown in Fig. 2. In this chapter, we will use the Make Your Own Flow Cytometer to illustrate the principles of flow cytometer operation. Full details about the system, including free video presentations of its assembly and use, can be found at https://www.cytometryworks.com.

2 Materials

1. A breadboard composed of one of the following:
   (a) A carbon fiber (CarbonVision GmbH, Unterschleissheim, Germany).
   (b) FDM (fused deposition manufacturing).
   (c) 3D printed with aluminum-threaded inserts: acetonitrile butadiene styrene (ABS) for somewhat rigid components, carbon fiber-infused ABS for highly rigid or nonreflective components, or thermoplastic polyurethane (TPU) for flexible components.

2. Laser: A Coherent Bioray cyan 488-nm direct diode laser emitting output attenuated to 5 mW (Coherent Laser, Mountain View, CA, USA).
3. A laser focusing lens (plano-convex 100-mm focal length) and steering prisms (5-mm flats: N-BK7 glass (Thorlabs, Newton, NJ, USA).
4. A flow cell: sources from BD Biosciences (San Jose, CA, USA).
5. Filters and dichroics: sources from Semrock/IDEX Health and Science, LLC (Rochester, NY, USA).
6. A photomultiplier tube (PMT) model H6779 (Hamamatsu Photonics, Shizouka, Japan).
7. Low-pressure air regulators (Dwyer Instrument, Michigan City, IN, USA).
8. Pneumatic switches and metal fluidic fittings (Pneumadyne/Norgren, Plymouth, MN, USA).
9. Power supplies: sources from Mean Well Enterprises Co. (New Taiwan City, Taiwan).
10. Azurite acquisition electronics (Darkling X, LLC, Los Alamos, NM, USA).
11. Kytos acquisition software (Darkling X, LLC).

More information can be found at https://www.cytometryworks.com or by contacting the author.

3 Methods

3.1 Instrument Assembly

While flow cytometers vary in design and complexity, they all have common features (Fig. 1). Flow cytometers require (1) a cell suspension, usually labeled with one or more fluorescent markers; (2) a method to introduce the cells into a narrow liquid stream, usually confined in a cuvette or flow cell; (3) a laser aligned with and focused on the cell stream, allowing "interrogation" of individual cells for both light scatter measurement and excitation of attached fluorescent probes; (4) optics, mirrors, and filters to capture and separate light scatter and fluorescent signals from the cells; (5) sensitive detectors to measure these signals; and (6) electronics, computer systems, and software to process, collect, and display the resulting data. All of these systems are illustrated below in the Make Your Own system.

A fully assembled Make Your Own system is shown in Fig. 2a, b. The system uses a baseplate where all the components are assembled (Fig. 2c), containing all the necessary attachment points for each component. Each component illustrates an important element in flow cytometer operation. Assembly of the system provides a good introduction to how flow cytometers operate.

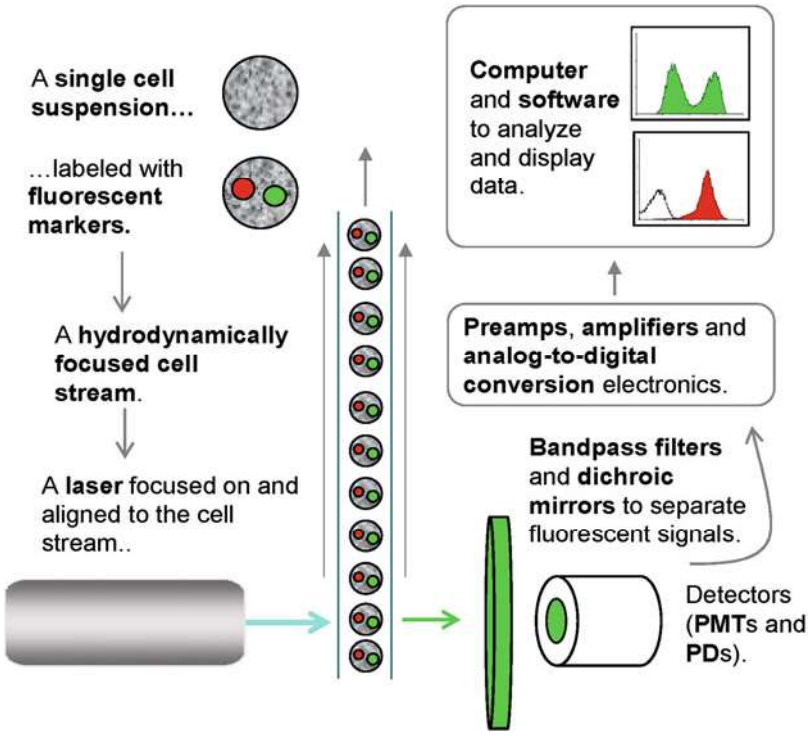

Fig. 1 Basic elements of a flow cytometer, including the characteristics of a cell population suitable for cytometry, generation of a hydrodynamic sample flow, interrogation of cells using a laser, detection of light scattering and fluorescence using collection optics, filters, dichroics and detectors, and processing and display of data

3.1.1 Lasers and Their Optics

Flow cytometers rely on lasers for both light scattering measurement of cells' physical characteristics and to excite any fluorescent probes incorporated into cells [5]. It is no coincidence that the development of the earliest flow cytometers and the invention of lasers both occurred within the same time period. Early flow cytometers relied on large water-cooled lasers with significant cooling and power requirements. Modern cytometers use solid-state lasers, including direct diodes and diode-pumped modules. These are smaller, easier to operate, and longer-lived than their earlier gas counterparts and are now the dominant laser type among commercial instruments. The Make Your Own system uses a small cyan 488-nm direct diode laser and is shown in Fig. 3a (installed on the cytometer) and Fig. 3b. Historically, the cyan 488-nm laser light (originally generated by argon ion gas lasers) was the primary excitation source for flow cytometers due to its ability to excite common fluorochromes such as fluorescein. This wavelength continues to be used in solid-state form. However, modern cytometers usually employ multiple laser sources, with red, violet, ultraviolet (UV), and yellow being common additions. These arrays of multiple lasers allow many fluorescent probes to be excited

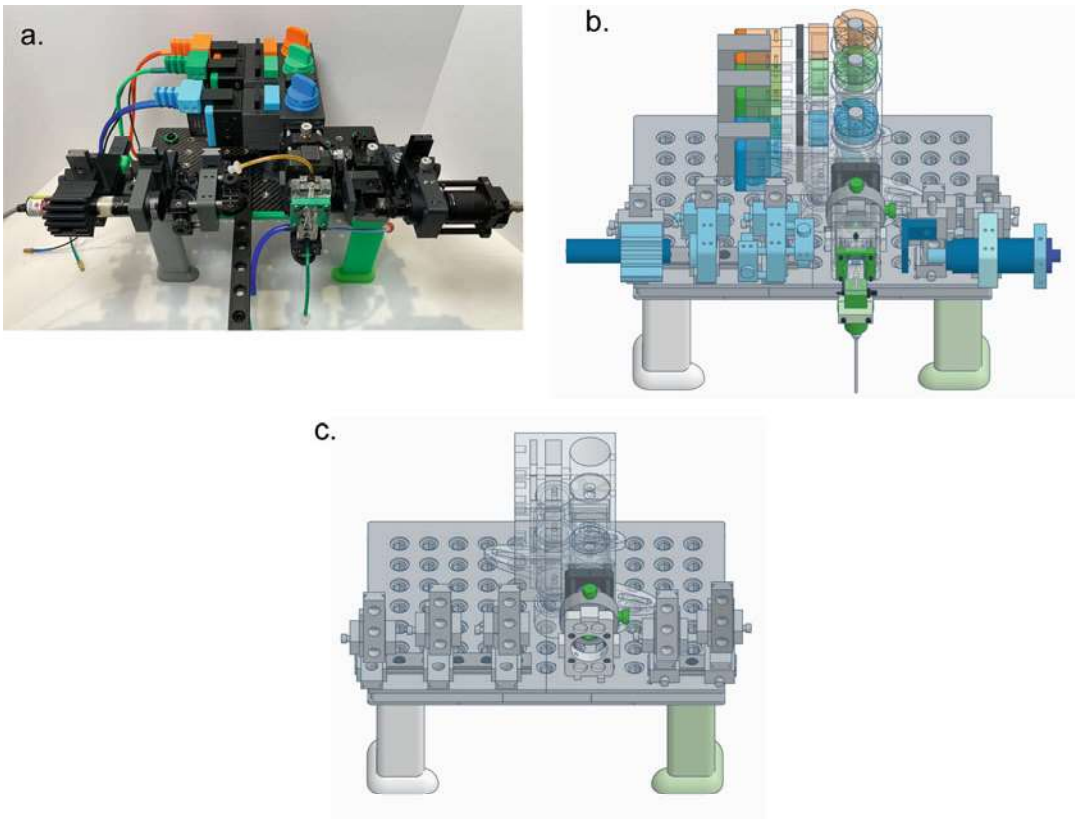

Fig. 2 (**a**) A fully assembled Make Your Own Flow Cytometer. (**b**) A CAD schematic of the fully assembled system. (**c**) A CAD schematic of the empty breadboard prior to construction

simultaneously. Lasers applicable to flow cytometry are usually single mode (with a circular or elliptical beam output), with good output stability and low noise levels.

The laser beam needs to be aligned with the center of a flow cell, thus allowing it to intercept the cell stream and excite the target cells. Lasers therefore need to be modified in terms of alignment, focus, and beam shape to optimally intercept the cell stream. Laser beam modification optics used in most flow cytometers are installed on the cytometer, as shown in Fig. 3a. Figure 3c shows a focusing lens used to reduce the beam diameter from >1 mm to <100 μm, occluding the cell stream and directing the maximum amount of laser energy onto the individual cells. In multi-laser instruments, a more complex achromatic lens is required to focus several laser wavelengths simultaneously. Figure 3d shows a beam-steering mechanism composed of two rotating flat prisms to translate the laser beam and align it with the cell stream. This combination of beam focus and alignment optics is necessary to maximize laser excitation and is found in virtually all flow cytometers (Fig. 3e).

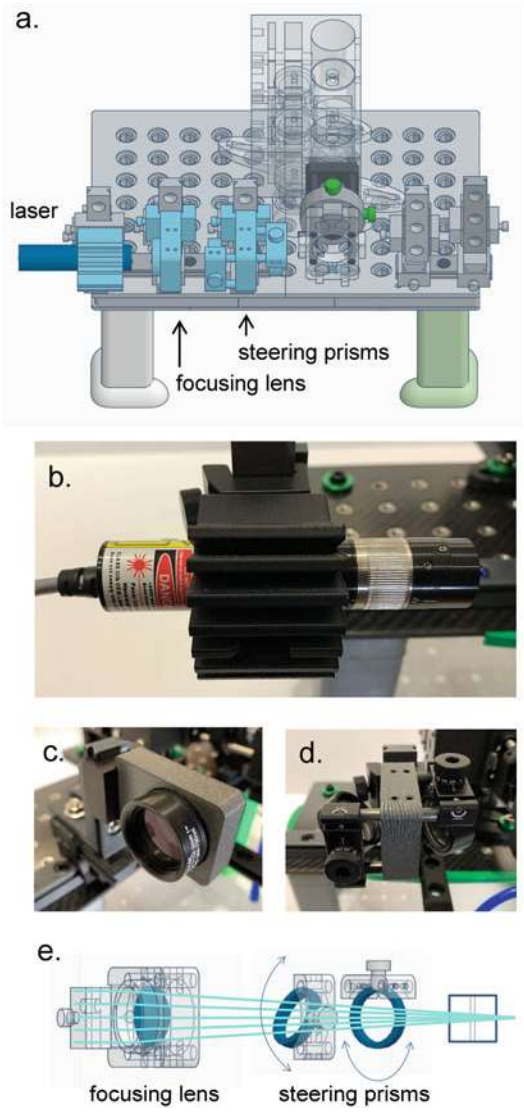

Fig. 3 (**a**) Installation of the laser, focusing lens, and steering prisms for beam focusing and alignment. (**b**) An installed cyan 488-nm direct diode laser. (**c**) A plano-convex focusing lens. (**d**) Steering prisms

Beam profile shaping is also an important element in correct sample stream interception.

Beam-shaping optics such as anamorphic prism pairs and beam-flattening optics (top-hat optics) can be used to modify the beam shape. An elliptical and flatter beam spot is usually more efficient at completely eclipsing the cell intercept with minimal vertical spill-over for multi-laser systems; specialized optics can be used to modify circular Gaussian beams to elliptical or flattened profiles for this purpose. These beam shape modifications also make

instruments more tolerant of stream fluctuations than can affect sensitivity. While not present in this simple instrument, they are often found in commercial systems.

3.1.2 Cell Delivery: The Flow Cell or Cuvette

Flow cytometers next require a mechanism to deliver a cell stream to the laser for scatter and fluorescence measurement. The most common system is a quartz flow cell or cuvette, with a narrow square or rectangular capillary (typically ~200 μm in width) aligned with a laser source. An example of a flow cell assembly containing a quartz cuvette is shown in Fig. 4a (installed on the cytometer) and Fig. 4b. This flow cell assembly is composed of an opening at the bottom, where the sample injection port tube (or the SIP tube) is installed, with a conical reservoir above it for introduction of the sheath buffer and the quartz cuvette above that (Fig. 4b). Cells are injected into this flow cell via the metal SIP tube, on which a sample tube containing cells is mounted (shown in Fig. 4c). The SIP tube injects the cells into the sheath stream, ultimately transporting into the quartz cuvette and through the focused laser beam (Fig. 4d). Sample injection can be achieved using positive or negative air pressure, a syringe pump, or a peristaltic pump. However, simply injecting the cells into the flow cell would not generate the highly focused cell stream required for uniform cell analysis; the cells would flow through the entire capillary and not be confined to a narrow path required for effective laser interception. Flow cytometers almost always employ hydrodynamic focusing to generate a highly confined cell stream. The cells are injected into an outer cylinder of moving liquid (the sheath stream). The conical reservoir below the flow cell shown in Fig. 4d is the entry point for the sheath flow in this flow cell unit. The laminar flow effect of the sheath stream pushes inward perpendicularly to the sample or core stream direction and focuses it to a narrow path at the center of the sheath flow. The sheath flow also exerts a parallel force on the sample stream, thus exerting control over its forward motion (illustrated in Fig. 4e). By carefully controlling the ratio between the sheath and sample flow pressure and keeping the sample pressure higher than the sheath pressure, an extremely narrow sample stream (typically less than 50 μm in diameter) with good stability can be generated, allowing the injected single cells to be efficiently and precisely aligned with and excited by the laser source. The outer sheath stream and the inner sample stream or core remain largely separated from one another in the cuvette, with little mixing. They both exit the flow cell assembly through the top and are sent to the instrument waste tank.

3.1.3 The Instrument's Fluidics

The sheath and sample (core) streams described above are generated by the instrument's fluidics, which push sheath buffer and sample into the flow cell. There are several mechanisms to do this this, including positive and negative pressure generated by air or

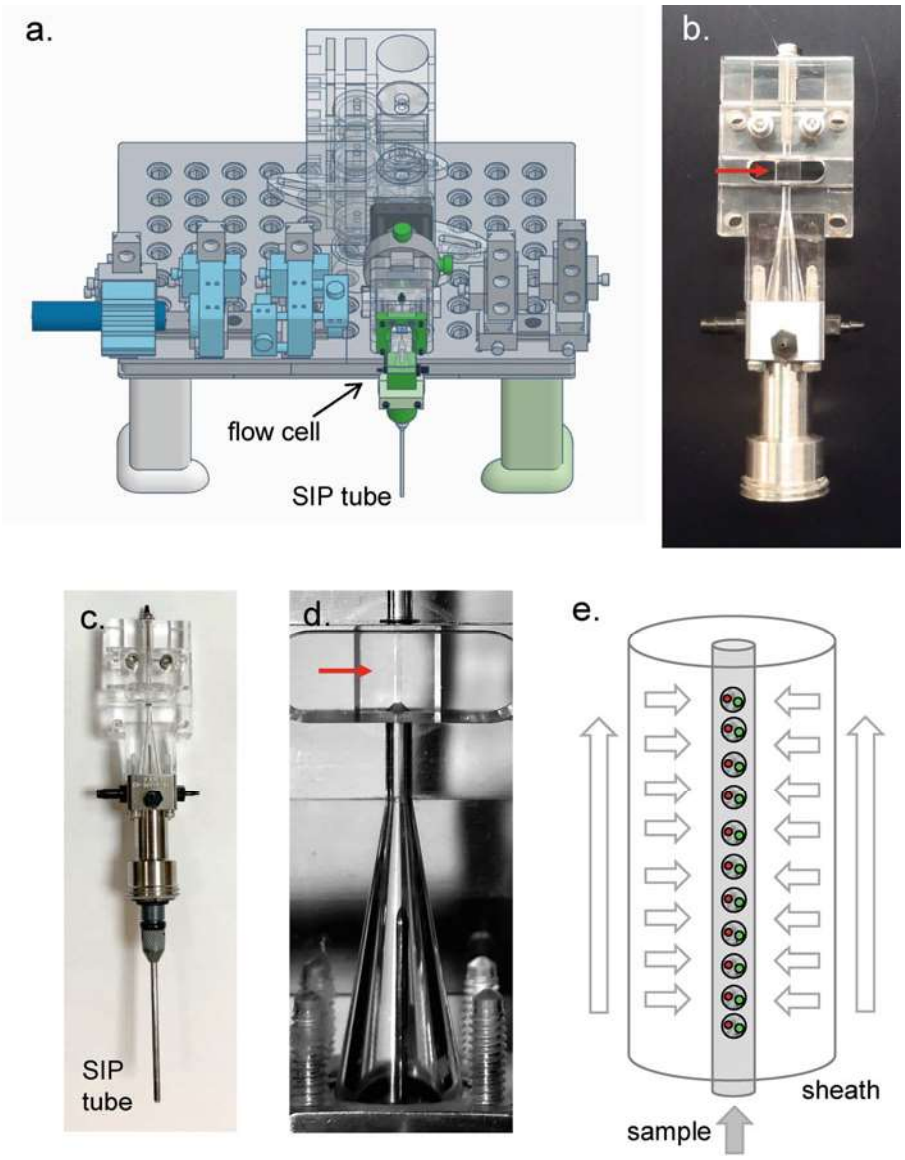

Fig. 4 (**a**) Installation of a flow cell/quartz cuvette and an SIP tube. (**b**) Details of the entire flow cell assembly, with the quartz cuvette indicated with a red arrow. (**c**) Flow cell assembly with the SIP tube inserted. (**d**) A close-up of the conical sheath reservoir immediately below the quartz cuvette. (**e**) An illustration of hydrodynamic focusing, with the cell stream (gray) confined and propelled by an outer cylindrical sheath stream (white)

vacuum pumps, syringe pumps, peristaltic pumps, or a combination of these mechanisms. Hydrostatic (positive pressure) systems are the simplest and historically the most common and are used in the Make Your Own system. The positive pressure system used in this system is shown in Fig. 5a. These systems use a sheath buffer tank, which is pressurized with an air pump. The sheath buffer is pushed

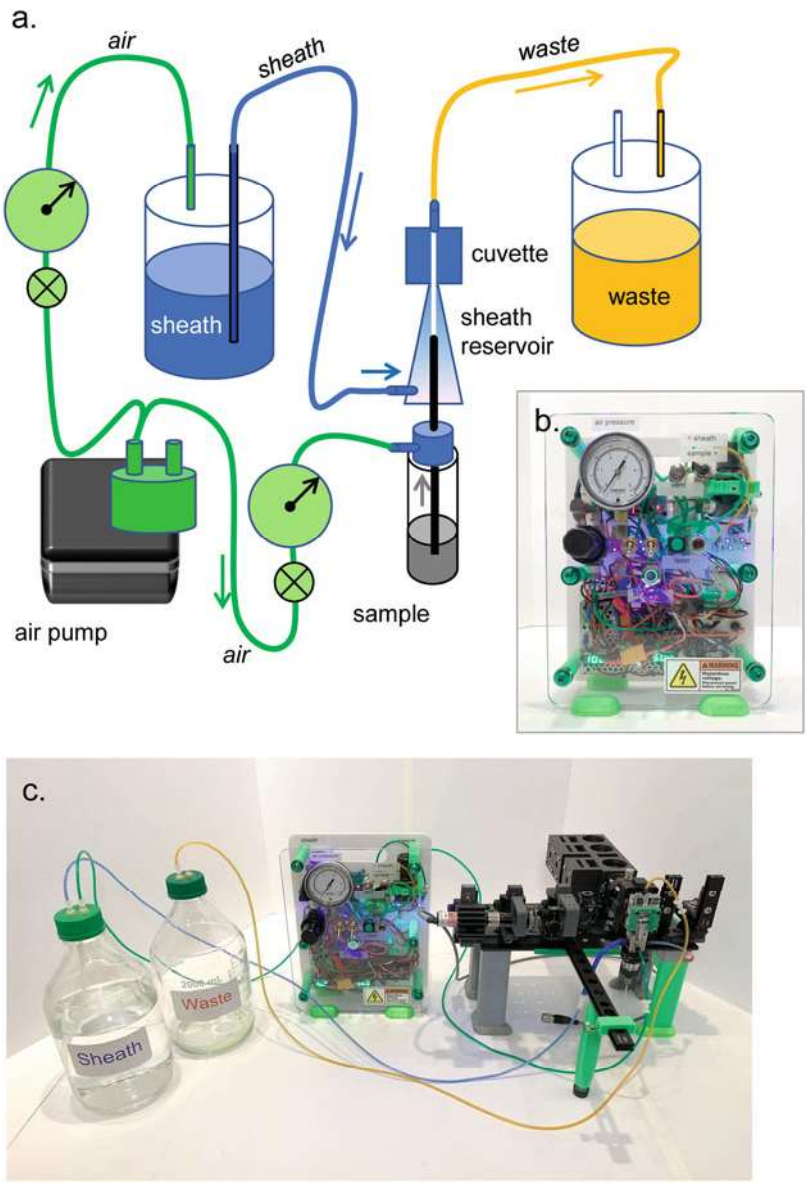

Fig. 5 (**a**) A schematic of the Make Your Own Cytometer fluidics, including an air pump, a pressure gauge and switch (green) sheath and a waste tank, a sample tube, and a flow cell. Air tubing (for both the sheath tank and sample tube) are indicated in green, the sheath buffer tubing in blue, and the waste tubing in orange. (**b**) A Make Your Own Cytometer pump console, containing air pumps, pressure gauges and switches, and power supplies. This unit supplies regulated air pressure to both the sheath tank and the sample tube. (**c**) A Make Your Own Cytometer connected to the air supply. Four tubes are shown (air tubing to the sheath tank and to the sample tube, sheath buffer tubing from the sheath tank to the flow cell assembly, and waste tubing from the flow cell to the waste tank)

into the flow cell assembly through a port connected to the conical reservoir below the quartz cuvette shown in Fig. 4c. This pressure can be controlled and adjusted. The same air pump also pressurizes a sample tube, pushing the sample into the capillary and the sheath flow. By varying the ratio between the sheath and sample pressure, a narrow sample or core stream can be achieved. Sample pressure must be slightly higher than sheath pressure, allowing the sample to successfully push against the sample flow and enter the capillary. After passing through the capillary, both the sheath and sample are ejected into a waste container.

Most analyzers use a fixed sheath pressure and allow adjustment of the sample pressure to increase or decrease the flow rate of the cell suspension. Increasing sample pressure does not increase the velocity of cells through the capillary. Instead, it increases the diameter of the sample stream as it pushes more strongly on the sheath stream. This has important implications for analysis; a wider sample stream will increase the probability that two cells will arrive at the laser intercept together. These coincident events can be misinterpreted as a single event. The laser beam might also not uniformly illuminate a wide sample stream. Maintaining a low sample pressure is therefore desirable for high-resolution cell analysis.

The Make Your Own system is configured for supplying both sheath and sample air pressure, as shown in Fig. 5c. The instrument's fluidics module (Fig. 5b) contains air pumps, pressure regulators, and switches, allowing sheath and sample pressure to be adjusted. Four connections are made between the fluidic system and the cytometer, including positive air pressure to the sheath tank and to the sample port below the flow cell (the green lines), a sheath buffer to the flow cell (blue), and a waste line from the top of the flow cell (orange). This is an extremely simple system. Commercial systems possess positive and negative feedback regulations to maintain sheath and sample pressure and ratios and are more complex.

While positive pressure systems are historically the most widespread mechanism for delivering cells in a flow cytometer, other methods for delivering sheath and sample pressure are also used. Negative air pressure (vacuum) systems are becoming more common. Many cytometers use syringe and peristaltic pumps to deliver the sheath buffer and/or the sample. These systems have more precise volume control and allow absolute cell counts to be calculated based on the flow rate of the pump mechanism.

3.1.4 Turning on the Sample Stream

With everything connected, the laser is turned on, the sheath and sample air pumps pressurized, and a sample tube containing yellow-green fluorescent particles (Polysciences YG 6-μm microspheres) is mounted on the SIP tube (Fig. 6a). The sheath pressure is set at approximately 2 psi and the sample pressure at about 2.5 psi

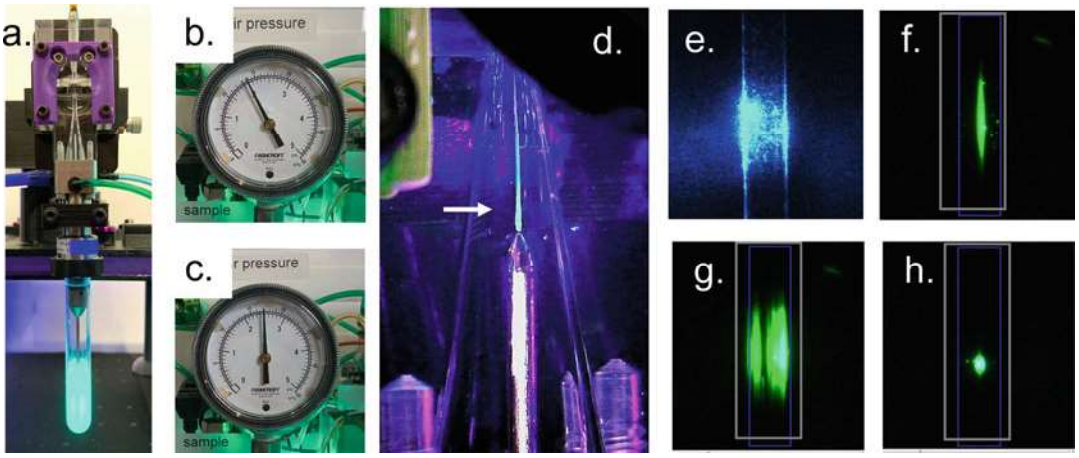

Fig. 6 (**a**) Flow cell assembly with the sample tube containing fluorescent microspheres mounted on an SIP tube. (**b**) A pressure gauge showing sheath pressure (~2 psi). (**c**) A pressure gauge showing sample tube pressure (~2.5 psi). (**d**) A close-up of the flow cell assembly's sheath buffer reservoir showing fluorescent beads injected into the sheath flow and quartz cuvette (illuminated by UV light). (**e**) A quartz cuvette illuminated by a 488-nm laser light (no notch filter block) showing cuvette walls. (**f**) A quartz cuvette with a notch filter block of 488-nm laser light and fluorescent microspheres passing through as sample. The hydrodynamic focusing should be noted. (**g**) A quartz cuvette with a notch filter block of 488-nm laser light and fluorescent microspheres passing through with no sheath flow (sheath tank depressurized). The lack of hydrodynamic focusing should be noted. (**h**) A quartz cuvette with better laser focusing on the fluorescent microsphere stream, resulting in a smaller beam spot

(slightly higher) (Fig. 6b, c). To successfully inject the sample into the sheath stream, the sample stream must be at a higher pressure to overcome the sheath flow. To illustrate sample flow and the effects of hydrodynamic focusing, two cameras are mounted on the Make Your Own system: one aimed at the conical sheath reservoir and the other at the quartz cuvette. These allow the user to visualize the sample stream both prior to cuvette entry and inside the cuvette. A notch filter that blocks cyan 488-nm light but allows other wavelengths through allows the sample to be seen while excluding the laser light.

Figure 6d shows the conical sheath reservoir with the SIP tube visible on the first camera. If illuminated by ultraviolet light, the sample stream is shown entering the sheath reservoir and is both confined by it and drawn toward the quartz cuvette (white arrow). This shows the dramatic effect of hydrodynamic focusing, which maintains a narrow stream diameter and channels it toward the quartz cuvette. The second camera provides magnified images of the quartz cuvette, as shown in Fig. 6e–g. The empty quartz cuvette illuminated by a laser light (no notch filter) is shown in Fig. 6e prior to sample injection. The vertical walls of the cuvette are visible. In Fig. 6f, the yellow-green fluorescent beads are run as a sample and the laser light is blocked with a notch filter. The walls

of the flow cell (now invisible) are marked in gray. Only the fluorescent beads are visible. The hydrodynamically focused bead stream is visible and is clearly confined to the center of the flow cell. Without sheath flow, the beads would not be confined to the center of the flow cell; they would not be uniformly excited by the laser, and many cells would arrive at the laser intercept simultaneously and unevenly, making single cell analysis impossible. This is shown in Fig. 6g in which the sheath flow has been stopped while continuing to inject the sample; the fluorescent bead stream fills the entire flow cell. Hydrodynamic focusing is therefore essential to flow cytometry and virtually all flow cytometers employ it.

Figure 6e–g shows laser illumination of the sample stream with a somewhat defocused laser. Proper focusing of the laser is shown in Fig. 6h, in which an extremely small beam spot has been achieved by adjusting the focusing lens. Proper laser focusing maximizes both excitation and sensitivity. An overly narrow focus can, however, result in low tolerance for stream perturbations, rendering the instrument focus unstable. Many commercial systems utilize their beam-shaping systems to slightly defocus the lasers and make their alignment robust while maintaining instrument sensitivity. More powerful lasers can also be used, although at a higher cost.

3.1.5 Light Collection, Dichroic Mirrors, and Filters

Once the laser interrogates the cell, the resulting signals must be collected by efficient light-collecting optics and separated by downstream dichroic mirrors and filters. The Make Your Own Cytometer utilizes an extremely simple optical configuration, analyzing side scatter and two fluorescent detectors using dichroic mirrors and narrow bandpass filters assembled at right angles (Fig. 7a). The quartz cuvette is coupled to a high numerical aperture light collection optic, which focuses the side scatter and fluorescent signals onto the instrument optical path. This optic is shown in Fig. 7b, positioned directly behind the cuvette and cell stream. These optics can be air-spaced in cell sorters or physically coupled using a numerical aperture-matched optical gel for analyzers. Modern flow cytometers often use fiber optics to transmit scatter and fluorescent signals to detectors.

Detection of laser light scatter and multiple fluorescent signals requires multiple optical elements to separate different emission wavelengths. This simple system uses two dichroic mirrors to separate the cyan 488-nm side scatter signal (a rough measure of cell optical density) from the two green and orange fluorescent signals. Dichroic mirrors are coated optics that allow light transmission above or below a certain wavelength while reflecting light below or above when positioned at a particular angle of incidence. The dichroic mirrors shown in Fig. 7c, d include a 510-nm long pass (LP) dichroic, transmitting light >510 nm and reflecting light <510 nm (a cyan 488-nm laser light constituting a side scatter

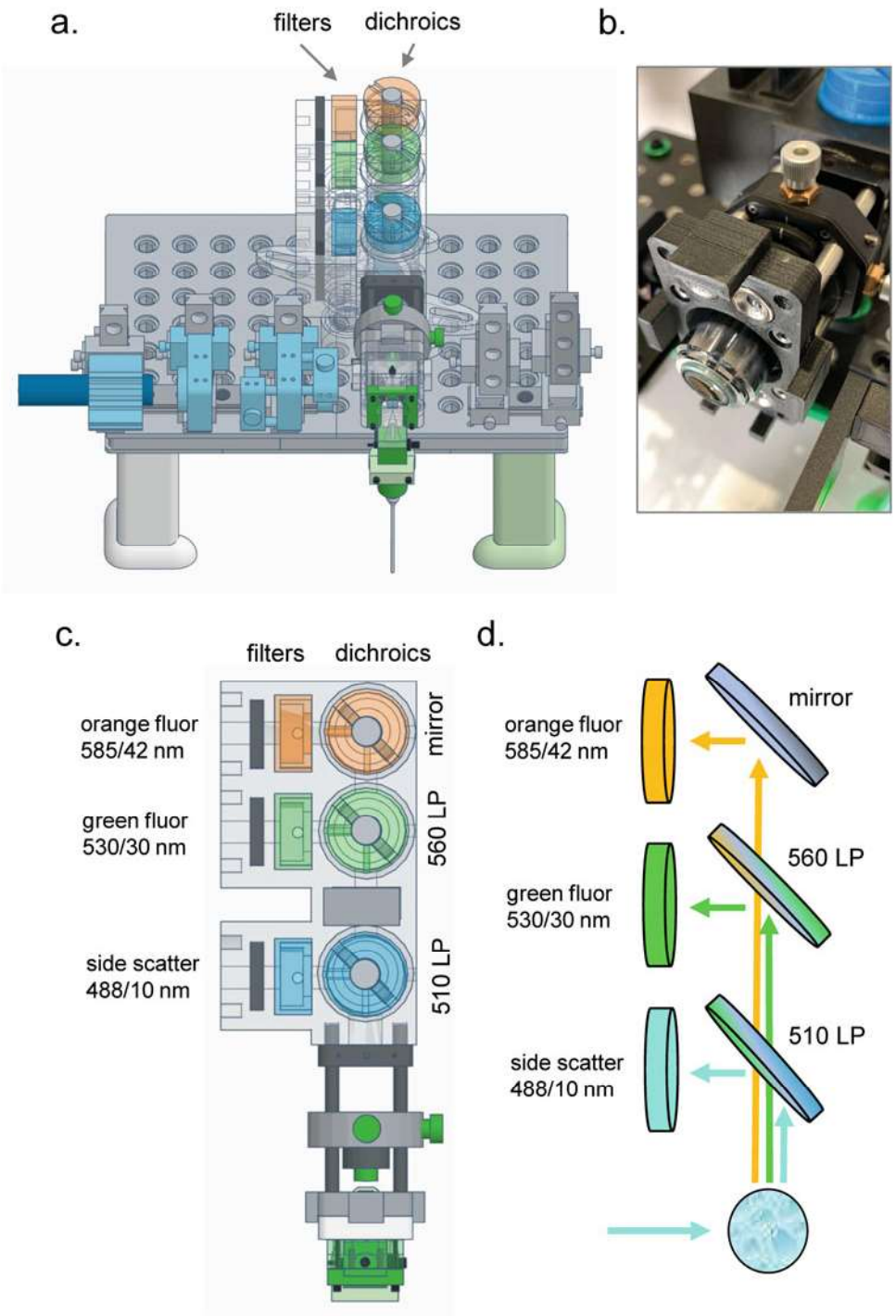

Fig. 7 (**a**) Installation of dichroic mirrors and bandpass filters for side scatter and green and orange fluorescence detection. Blue, 510-nm long-pass dichroic and 488/10-nm bandpass filter; green, 560-nm long-pass dichroic and 530/30-nm bandpass filter; orange, 100% mirror and 585/42-nm bandpass filter. (**b**) A close-up of a high numerical aperture light collection optic for side scatter and fluorescent signals. (**c**) A CAD schematic of the optical layout with dichroic mirrors and bandpass filters indicated. (**d**) A schematic of the optical layout with dichroic mirrors and bandpass filters shown

signal, a rough measure of cell optical density). The resulting transmitted light above 510 nm is then separated again using a 560-nm long-pass (LP) dichroic. The reflected light is between 510 and 560 nm, which would include the green-emitting probe fluorescein. The remaining light above 560 nm would include orange-emitting fluorochromes like phycoerythrin. Both green and orange signals are therefore reflected toward detectors for these fluorochromes. These dichroics mirrors are specified for use at 45° angles of incidence, critical for their wavelength transmission and reflectance specifications. More compact optical configurations can be constructed using dichroics with smaller angles of incidence, including the linear circular detector clusters found in many high-dimensional instruments. However, the same basic optical principles in more complex traditional cytometers are employed as in this extremely simple model.

After the initial separation by dichroic mirrors, bandpass filters are then used to further filter each fluorescent probe emission. Green and orange bandpass filters are shown in Fig. 7c, d. Their values (488/10 nm, 530/30 nm, and 585/42 nm) correspond to the peak transmission wavelength and the width of their transmission range. Most flow cytometers make extensive use of both dichroic mirrors and bandpass filters to separate and filter emission light from any fluorochromes simultaneously. Careful selection of optics allows large numbers of fluorescent probes to be analyzed simultaneously.

3.1.6 Side Scatter and Fluorescence

Scatter and fluorescent cell signals differ in strength; the laser scatter signals can be extremely strong, while fluorescent signals excited by the laser can be extremely weak. Different technologies are required to detect these signals. Weaker signals typically require sensitive photon detectors. Photomultiplier tubes (PMTs) are historically the detectors of choice for fluorescent signals. They are extremely sensitive photon detectors; some PMTs can detect a single photon of light. While not this sensitive, the PMTs on flow cytometers can detect as few as a few thousand molecules of some fluorescent probes.

The Make Your Own Cytometer has three PMTs for side scatter and fluorescence detection, shown mounted on the instrument in Fig. 8a, c. Individual examples are shown in Fig. 8b. PMTs require high voltage for signal amplification; adjustment of a PMT's sensitivity and dynamic range are made by controlling the voltage flowing to the PMT. The cables shown connected to the PMTs in Fig. 8c both supply this voltage and transmit the resulting scatter and fluorescent signals to the instrument electronics. Like much of the technology in modern flow cytometers, PMTs are now relatively small in size and can be incorporated in large numbers into cytometers capable of high-dimensional analysis. Until recently,

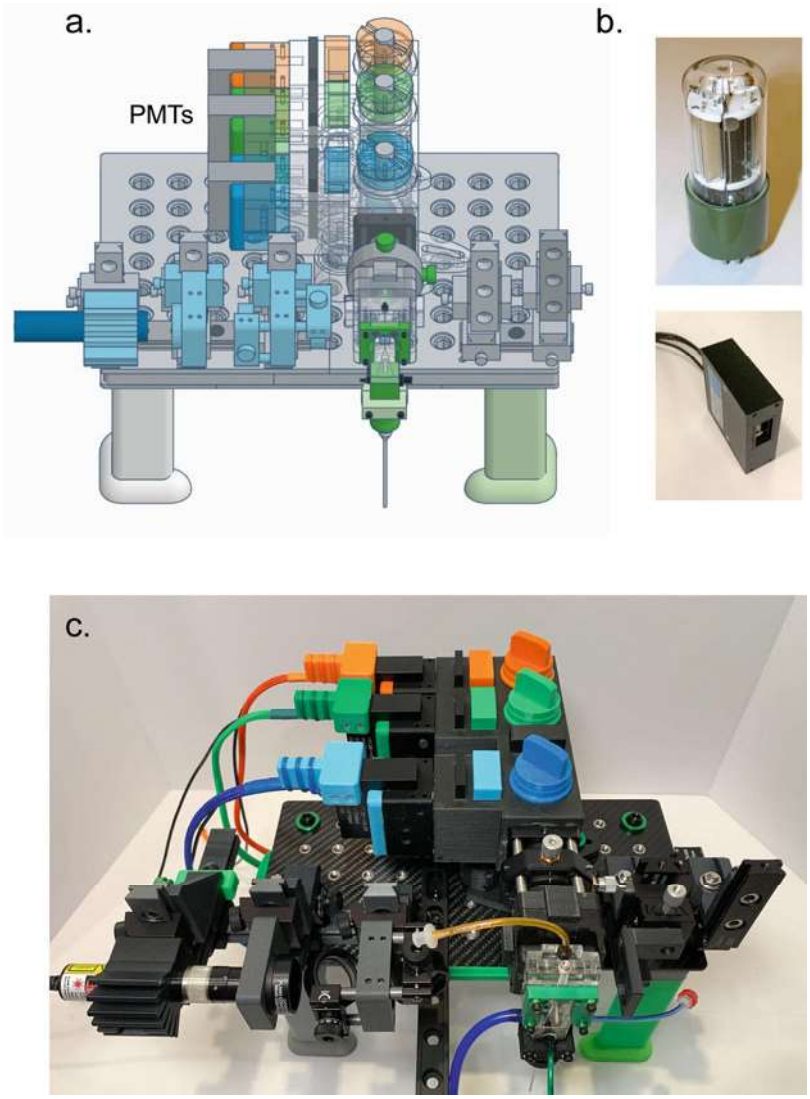

Fig. 8 (**a**) Installation of photomultipler tubes on the Make Your Own Flow Cytometer. (**b**) Examples of PMTs, including a side window H1477 PMT (upper photo) and an integrated H6779 PMT (lower photo) as used on the instrument. (**c**) PMTs installed on the instrument, with voltage and signal cables visible

PMTs have been the only practical light detection technology sensitive enough to detect the low levels of fluorescence often seen in biological samples. Newer solid-state technologies like avalanche photodiodes (APDs) and silicone photomultipliers (SiPMs) are starting to achieve similar sensitivities and are now being incorporated into commercial instrumentation. Their durability, small size, and low cost are also contributing to improved instrument designs.

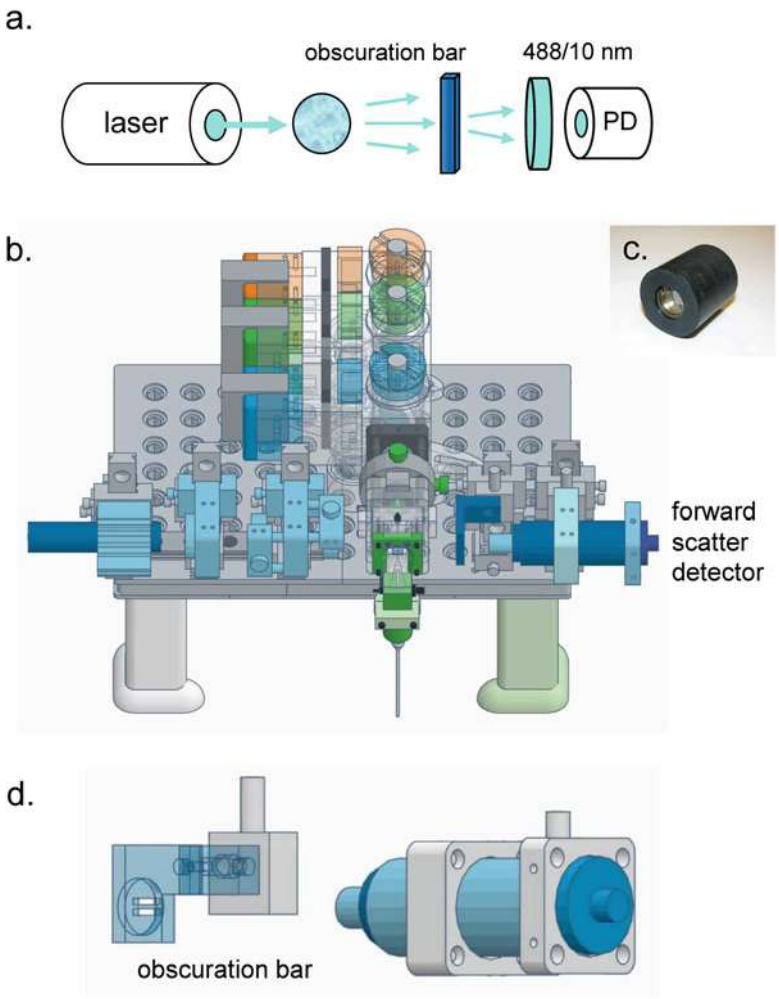

Fig. 9 (**a**) A schematic of a forward scatter detector, including obscuration or blocking bar, a 488/10-nm bandpass filter, and a photodiode (PD). (**b**) Installation of a forward scatter detector on the Make Your Own Cytometer. (**c**) A CAD diagram of a forward scatter detector, including an obscuration bar, an optical assembly, including collimation lenses and a pinhole, and a PD

3.1.7 Forward Scatter

Unlike side scatter and fluorescence, forward scatter is a much stronger signal. Forward scatter is scattered laser light detected at 180° from the incoming laser light and is a rough measure of cell size (Fig. 9a). Photodiodes (PDs), either passive (unpowered) or with a low voltage bias, are often used to detect this much stronger signal, with an example shown in Fig. 9c. A simple forward scatter detector is employed in the Make Your Own system, including collection optics, a pinhole, and a photodiode, as shown in the Make Your Own system in Fig. 9b and in the schematic in Fig. 9d. A flat strip of metal (called an obscuration or a blocker bar) is positioned in front of the detector and blocks direct laser

light, allowing only scattered laser light to reach the detector (Fig. 9d). While PDs are mostly employed for forward scatter detection, flow cytometers designed for small particle analysis often employ PMTs for increased submicron scatter sensitivity.

3.1.8 Electronics

The resulting signals are then processed by the instrument electronics and digitized for transmission to the system's computer. Early cytometers heavily relied on signal processing in analog electronics, converting the data to digital only as a last step. Modern systems use integrated digital electronics for initial signal processing, allowing faster and more accurate data analysis. Modern systems also make use of conventional computers for data acquisition and place much more reliance on the computer system for signal processing. The Make Your Own Cytometer has preamplifiers that initially amplify the scatter and fluorescent signals and (for photomultipliers) invert their peaks to positive signals and an amplifier and a digitizer unit (the Azurite module, designed and built by Mark Naivar, Los Alamos National Labs/Darkling X, LLC) to further amplify the signals and convert them from analog to digital (Fig. 10a, b). Photomultiplier power is also applied through this module. The amplifier/digitizer module is connected to a computer, which controls detector voltages and gains and manages data acquisition and display. A fully assembled instrument is shown in Fig. 10c.

3.2 Data Acquisition and Analysis

All commercial cytometers have proprietary software packages that allow control of the instrument, including detector sensitivity adjustment, as well as data acquisition and display. There are also a variety of third-party software packages that allow analysis of previously acquired data. These software packages are often more powerful for post-acquisition data analysis and are often preferred for this purpose but cannot initially acquire data. The data acquisition software utilized by the Make Your Own system is Kytos (also designed and built by Mark Naivar, Los Alamos National Labs/Darkling X, LLC), which can adjust detector voltages and gains and can display data in one- and two-parameter plots and also as oscilloscope traces. Acquisition of yellow-green microspheres illustrated above is shown in Fig. 11. A two-parameter scatterplot shows forward versus side scatter, with both parameters displayed on linear scales. One-parameter histograms for green and orange fluorescence are displayed on logarithmic scales, typical for fluorescence distributions with large changes in magnitude such as leukocyte immunophenotyping. Controls for photomultiplier voltage adjustment and amplifier gains are visible, as are detector thresholds and other critical instrument controls. Once detector voltages and gains are set by the user, the data are acquired and stored for subsequent analysis, usually with an analysis-only program.

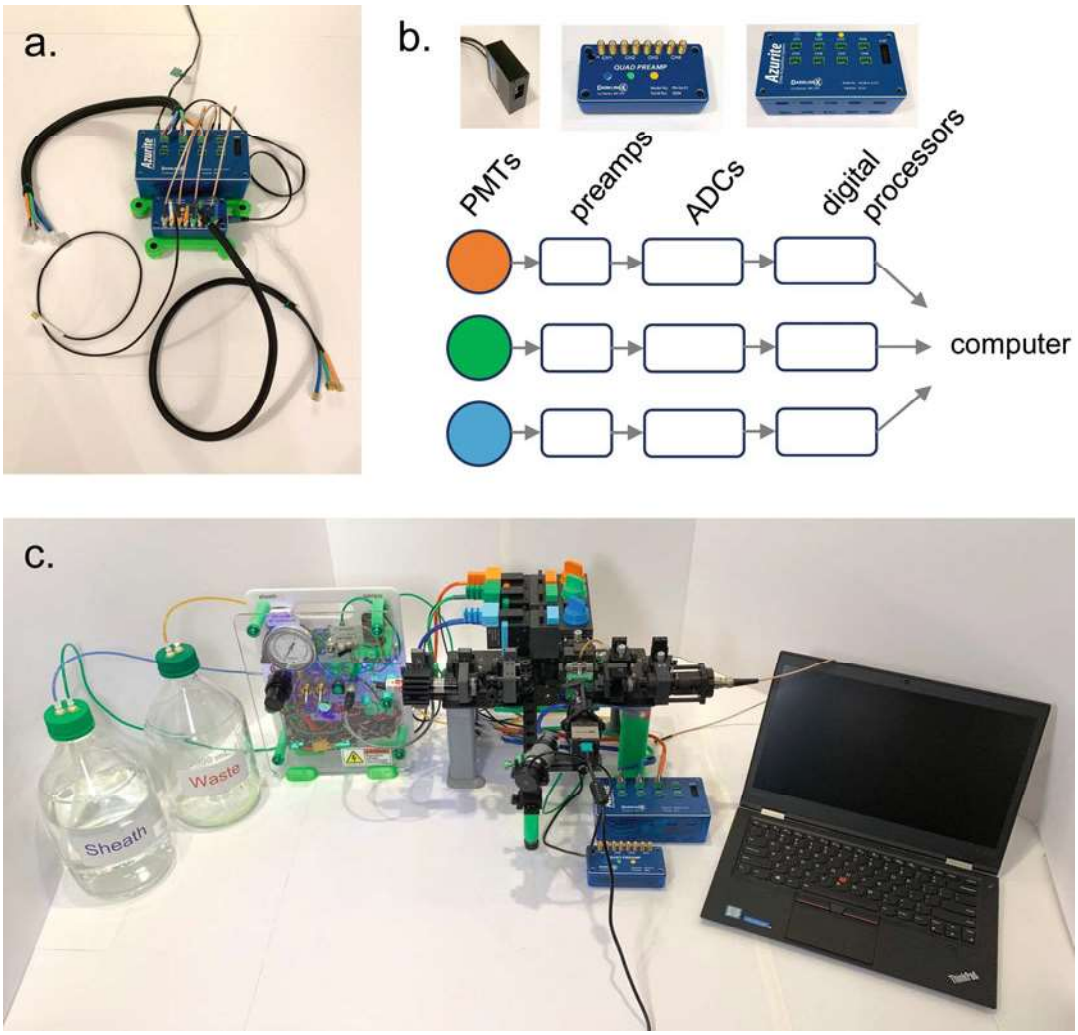

Fig. 10 (**a**) Electronics for the Make Your Own Cytometer, including a preamplifier and an Azurite amplifier/digitizer unit. (**b**) A schematic of cytometer electronics on modern cytometer systems, including a preamplifier, amplifiers, analog-to-digital converters (digitizers), and digital signal processors (DSPs). DSPs are now used in high-dimensional systems with high data processing requirements. (**c**) A fully assembled Make Your Own Cytometer system

3.3 The Fully Assembled Flow Cytometer: Optics, Fluidics, Electronics, and Software

Like many biomedical instruments, a flow cytometer is an integration of photonic and optical devices and components, with fluidic systems providing air and liquid pressure and flow control, microfluidic devices such as quartz cuvettes providing the means for optical analysis, and electronics and computer systems for rapid analysis of single cells for a variety of physical characteristics. The Make Your Own Cytometer illustrates an example of these systems working together to produce a functional instrument. Most of the technological phenomena required for flow cytometry illustrated here can be provided in multiple ways (such as different ways to

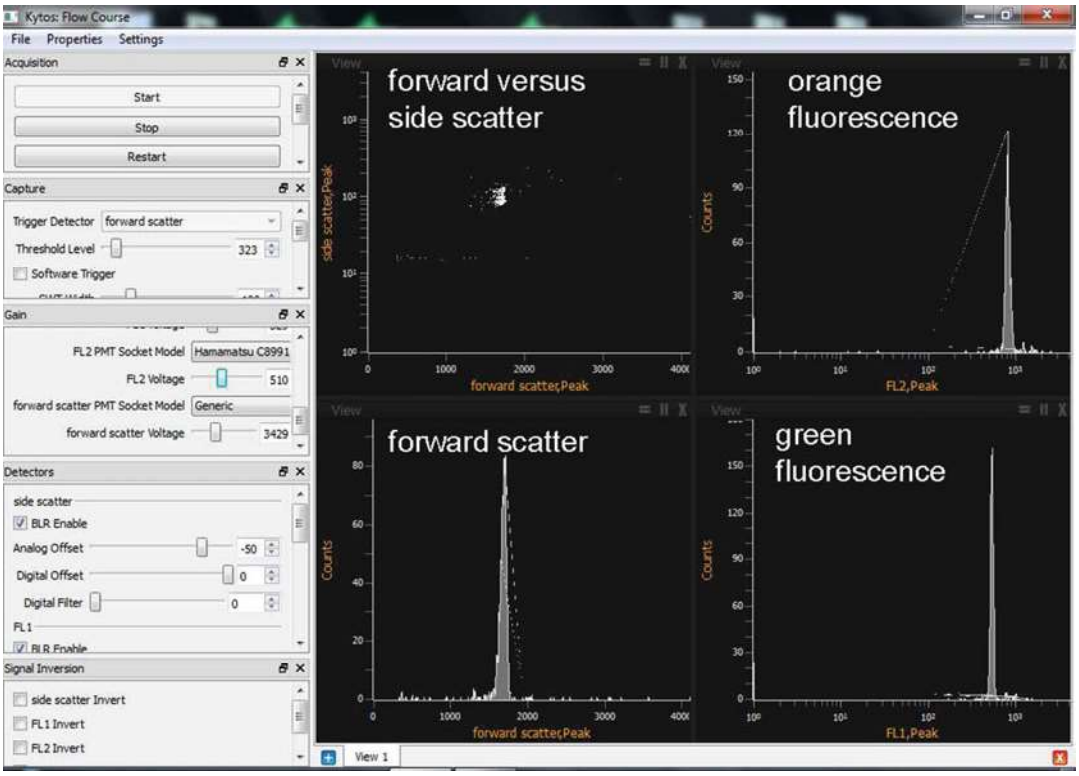

Fig. 11 (**a**) Kytos data acquisition software. A dot plot or scatterplot for forward versus side scatter (linear scaling), histograms for forward scatter (linear), and green and orange fluorescence (logarithmic scaling) are shown. Instrument controls, including start/stop acquisition, threshold and detector voltages, and gains, are visible

produce air or liquid pressure, photon detection, etc.), but almost all cytometers rely on the basic principles described above.

The Make Your Own Cytometer is an extremely simple instrument designed for teaching purposes, with only one laser and two fluorescent parameters. Modern flow cytometers are typically equipped with at least three laser wavelengths and as many as seven or more. Synthetic fluorescent probe development has resulted in large arrays of spectrally distinguishable dyes excited at most wavelengths in the visible spectrum, particularly with violet and ultraviolet laser sources [3, 4]. So-called high-dimensional flow cytometers are capable of detecting 20–40 fluorochromes simultaneously [5]. However, the overall optical designs are ultimately based on the optical principles described above, differing only with the addition of more lasers, detectors, and optics. High-dimensional flow cytometry has allowed deep profiling of the immune system, detecting and correlating many extracellular and intracellular protein markers and identifying dozens of distinct

leukocyte subsets. High-dimensional analysis using traditional optics employed by the Make Your Own Cytometer has allowed the analysis of over 30 distinct markers simultaneously. This is probably the upper limit of traditional systems.

Improvements in optical technology are now expanding the limits of flow cytometry even further. Full spectral flow cytometry collects the entire spectra of multiple fluorescent probes rather than simple bandwidths as described above. This technology allows fluorochromes with similar spectral characteristics to be used together with less overlap than with traditional optical methods. At the time of this writing, 40 or more markers can now be analyzed using spectral cytometry. Nevertheless, the relatively simple principles described above are still the basis of operation for all flow cytometers.

Acknowledgments

The Make Your Own Cytometer system is a not-for-profit and volunteer effort aimed at educating biomedical scientists in the inner workings of flow cytometers. Many individuals have made contributions to the design, construction, and dissemination of this system. Thanks go to John Martin, Mark Wilder, and James Jett, formerly of the National Flow Cytometry Resource (NFRC) in the Los Alamos National Laboratories Biosciences Division, and the creators of the original Building Your Own Flow Cytometer concept and instrument, who provided encouragement and technical expertise in building the Make Your Own Cytometer. Their system is still educating young scientists today. Scott Cram, also formerly of LANL NFRC, was a crucial partner in designing the system. Mark Naivar, formerly of LANL and NFRC, founder of the Darkling X, LLC, and currently at Miltenyi Biotec (Germany) designed and built the Azurite electronics and Kytos acquisition software.

As of this writing, the system has been used in international flow cytometry workshops in over 18 countries. This would not have been possible without the assistance of the International Society for Advancement of Cytometry (ISAC) and the ISAC Live Education Task Force, founded by Dr. Awtar Krishan-Ganju (formerly of the University of Miami School of Medicine) and currently managed by Zosia Maciorowski (formerly of the Institute Curie, France). While too numerous to list, we are deeply grateful to the organizers of the many international workshops in six continents, who have hosted the system and provided hundreds of students with the opportunity to build their own flow cytometer.

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