We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

8

Multiplexed Cell-Counting Methods by Using Functional Nanoparticles and Quantum Dots

Hoyoung Yun[1], Won Gu Lee[2] and Hyunwoo Bang[1]
[1]*School of Mechanical and Aerospace Engineering, Seoul National University*
[2]*Department of Mechanical Engineering, Kyung Hee University*
Republic of Korea

1. Introduction

This chapter mainly deals with investigation and development of intensity-based cell-counting methods using fluorescent silica nanoparticles (SiNPs) and quantum dots (QDs) for differential counting of leukocytes. The proposed cell-counting methods enable us to simultaneously measure multiple subsets of human blood cells using a single detector without fluorescence compensation due to an inherent signal overlap of emission spectra from multiple fluorescent labels. At the beginning of the chapter, brief history and theoretical background of multicolor flow cytometry and previous intensity-based cell-counting methods are reviewed. Subsequently, motivation and objectives of the proposed methods are introduced with current issues in this field.

Antonie van Leeuwenhoek (Holland, 1632-1723) is the first person who observed blood cells and micro-organisms in suspension using the simple microscope (~300 X). As microscopy techniques have rapidly developed, the first commercial microscope with ultraviolet (UV) was presented by Carl Zeiss (Germany) in 1904 and the phase-contrast microscope that allowed for the study of colorless and transparent biological materials were invented in 1932. Meanwhile, George Gabriel Stoke (1819–1903) first described a fluorescence difference between excitation and emission spectra known as the Stokes shift in the Mid-1800's. A fluorescent antibody technique developed by Albert Coons (1912–1978) in 1941, who labeled antibodies with fluorescein isothiocyanate (FITC), thus he gave birth to the field of immunofluorescence. From Mid-1900's, scientists began to interest in automated cell-counting techniques, not just in observation of cells. Moldovan described the first flow cytometer concept using glass capillary tubes mounted on a microscope stage (Moldavan, 1934), although this device could not measure meaningful cell-signals because of capillary blocking and interference of signals by using narrow tubes. When wider tubes were used, the device could not count cell population. In 1947, a photoelectric counter, which uses light source and photomultipliers (PMTs), was developed and this device is the first working flow cytometer (Gucker Jr et al., 1947). To test the efficiency of gas mask filters against particles, the device used filtered air to carry and constrain the sample particles. A hydrodynamic focusing concept for reproducible delivery of cells suspended in a fluid was introduced by Crossland-Taylor in 1953. Using this device, accurate counts of blood cells were obtained (Crosland-Taylor, 1953). The first impedance-based flow cytometer by using

the Coulter principle was developed in 1953 (Coulter, 1953). This principle was used in the first demonstration of cell sorting in 1965 (Fulwyler, 1965). The first commercial fluorescence-based flow cytometry device (ICP 11, Partec, Germany) reached the market in 1969. Fluorescence Activated Cell Sorter (FACS) was developed by Leonard A. Herzenberg (Herzenberg et al., 1976) and firstly commercialized by Becton Dickinson (BD, USA) in 1974.

In 1977, the first multi-parametric cell counting method using monoclonal antibodies (Loken et al., 1977), which was called a two-color immunofluorescence method, was developed by Leonard A. Herzenberg and his colleagues. Subsequently, they described a three-color immunofluorescence detection system in 1984 (Parks et al., 1984) and this was beginning of the multicolor world of flow cytometry.

In 1934	Moldovan described the first flow cytometer (Moldavan, 1934)
In 1947	The photoelectric counter was developed (Gucker Jr et al., 1947) The first working flow cytometer
In 1953	Development of the concept of hydrodynamic focusing (Crosland-Taylor, 1953) The first impedance-based flow cytometry device (The coulter counter) (Coulter, 1953)
In 1965	The coulter principle was used in the first demonstration of cell sorting (Fulwyler, 1965)
In 1969	The first commercial fluorescence-based flow cytometry device was developed (ICP 11, Partec, Germany)
In 1974	The Fluorescence Activated Cell Sorter (FACS) was developed by Leonard Herzenberg and first commercialized by Becton Dickinson (BD) (Herzenberg et al., 1976)
In 1977	Two-color immunofluorescence using monoclonal antibodies and FACS was demonstrated (Loken et al., 1977) The fluorescent antibody technique (immunofluorescence) developed by Albert Coons (Coons et al., 1941), who labeled antibodies with fluorescein isothiocyanate (FITC)
In 1984	Three-color analysis: beginning the multicolor world of flow cytometry (Parks et al., 1984) Robert Murphy developed FCS 1.0 file standard (Murphy et al., 1984)

Table 1. Important developments in flow cytometry and multicolor immunofluorescence

The ability to simultaneously measure multiple parameters is the most powerful aspect of flow cytometers and enables a wide range of applications, including clinical applications and research applications. Recently, flow cytometers are the most commonly used automated cell counting and sorting devices for analyzing particles, beads or cells suspended in a fluid stream (Laerum et al., 1981, Shapiro, 1983). It has been widely applied in multi-parametric studies on the physical and/or chemical characteristics of cells, leukocyte differentiation for cell based diagnostics, and immunoreaction based on micro beads (Brando et al., 2000, HOUWEN, 2001). These applications require multi-parametric information from multiple cytometers or a single cytometer equipped with multiple photomultiplier tubes (PMTs) to simultaneously detect target samples tagged with fluorescent dyes having different emission wavelengths (Janossy et al., 2000, Glencross et al., 2002, Janossy et al., 2003). More recently, a flow cytometer equipped with multiple light

sources and multiple detectors that can measure up to 16 optical parameters at the same time has been developed (Cottingham, 2005) and new methods to measure even more parameters have been suggested (Darzynkiewicz et al., 1999, Perfetto et al., 2004, Kapoor et al., 2007). Such developments can significantly enhance the reliability of cell based diagnostics and even make it possible to develop new diagnostic methods using the information given by the additionally acquired parameters.

Similarly, in parallel to developing the high performance flow cytometers requiring multi-parameter detection capabilities, portable flow cytometers have been recognized as an important tool for particular applications such as HIV/AIDS screening in developing countries and regions with limited medical facilities and resources (Cohen, 2004, Bonetta, 2005, Lee et al., 2010). Several foundations have provided support to ensure sustainable access to $CD4^+$ T-cell testing for developing countries and many researchers have made effort to develop CLIA (Clinical Laboratory Improvement Amendments)-waived flow cytometry or POC (Point-of-care) cell counting method.

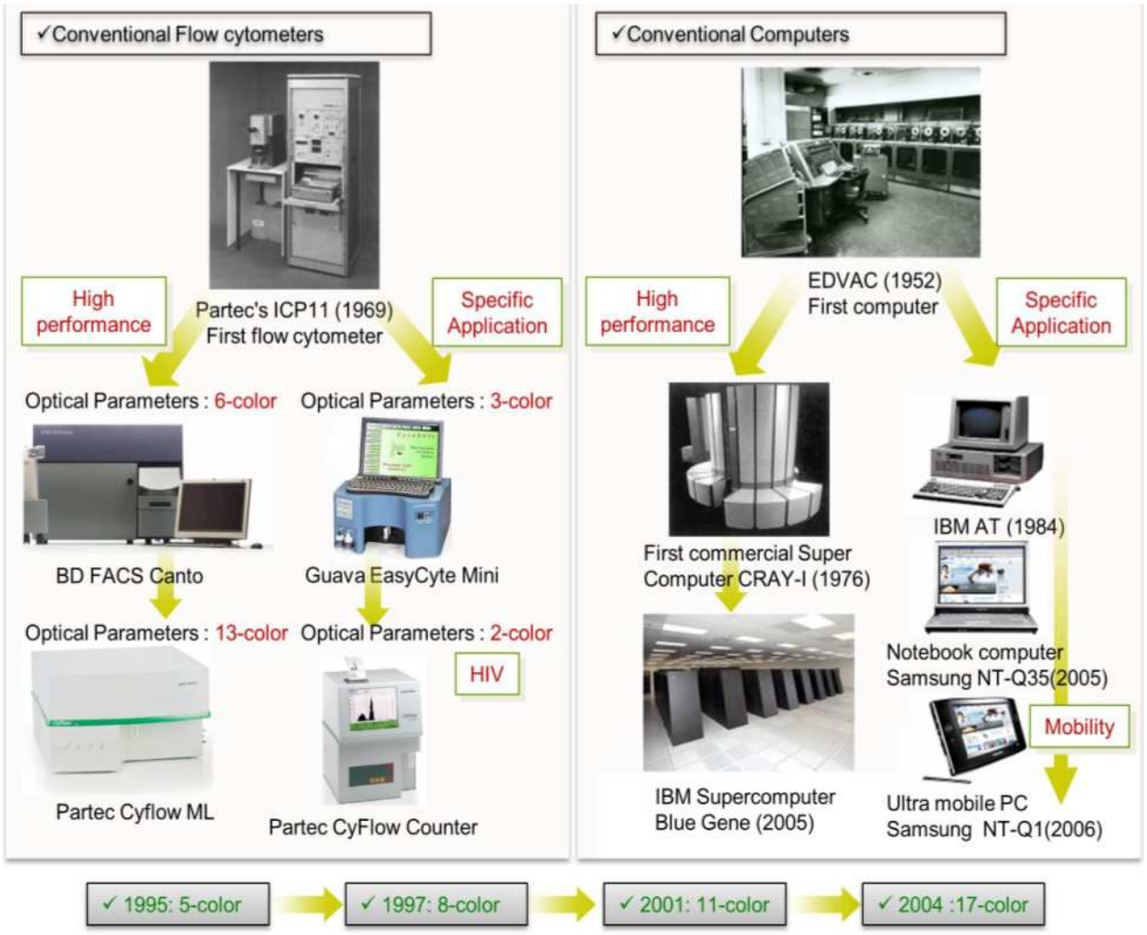


Fig. 1. The key trends of development of flow cytometers. Like computers, both of the high performance flow cytometer and the inexpensive portable flow cytometer have their own important role. (Figure sources from partec.com, bd.com, guavatechnologies.com, Samsung.com, ibm.com)

Since the invention of the first computer (EDVAC, 1952), there are two trends in history of development of computers: super computers for high performance and personal computers for mobility. In the same manner, flow cytometers will have been developing in two types: high performance flow cytometers for multi-parametric cellular analysis and inexpensive portable flow cytometers for point-of-care applications.

Multiplexed cell-counting methods in this chapter could be applied to both of high performance applications for measurement of multiple parameters on cells and point-of-care applications by using portable flow cytometers. The ability of these intensity-based cell counting methods to simultaneously measure multiple parameters by using single detector enables us to increase the number of detectable parameters per detector without fluorescence compensation. Therefore, conventional flow cytometers can detect more parameters without increase of detectors and portable flow cytometers can minimize the number of detectors.

2. Multicolor flow cytometry

2.1 One-color immunofluorescence and fluorescence dyes

An immunofluorescence staining is a technique used for analysis of biological samples. This technique allows detection of specific antigens or proteins by binding an antibody conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC) (Coons et al., 1941). For example, a CD4 antigen used for a HIV/AIDS screening is one of the most famous cell surface antigens of leukocytes. Biological samples, such as cells and tissue sections, stained by immunofluorescence can be analyze by fluorescence microscopes, confocal microscopes, or automated cell analyzers including a flow cytometer (Loken et al., 1977, Ledbetter et al., 1980).

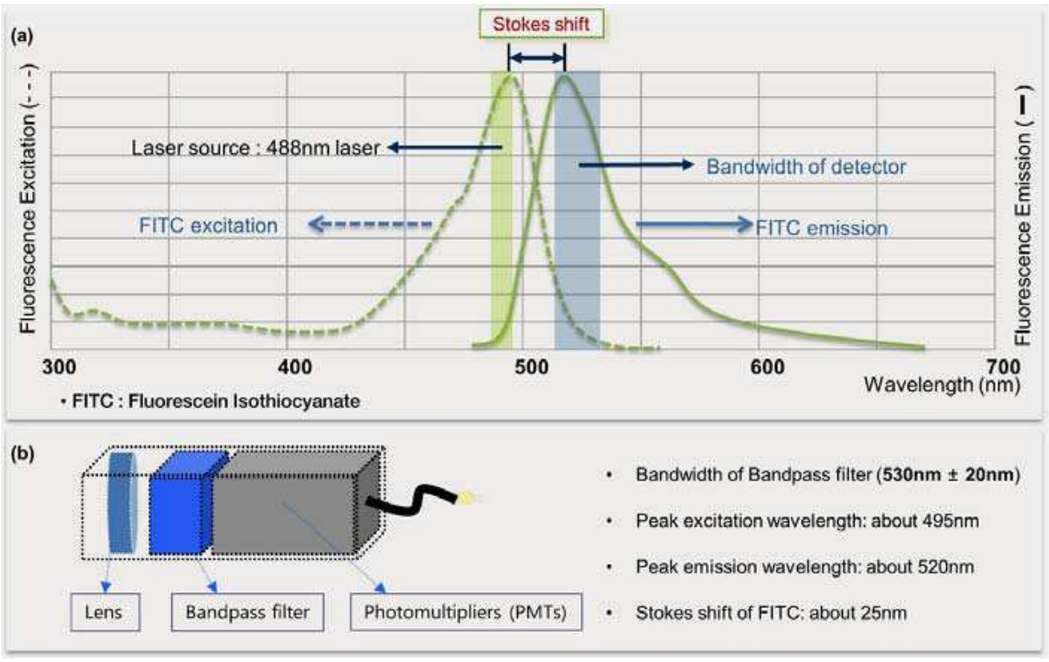


Fig. 2. Example of one-color immunofluorescence and graph of the Stokes shift of FITC

Basically, the immunofluorescence uses the Stokes shift which is a fluorescence difference between a peak excitation and emission wavelengths of the same electronic transition. As shown in Fig. 2, the peak excitation and emission wavelength of FITC is approximately 495nm and 520nm, respectively. Therefore, the Stokes shift of FITC, which is the most common used fluorescence dye in 1-color immunofluorescence, is 25nm. By using a 488nm excitation light source, an optical bandwidth filter (530nm ± 20nm), and a detector (PMTs), we can detect and count a desired marker in cells or biological samples.

Desirable fluorescence dyes for flow cytometry have several properties as follows: 1. they have biologically inertness, which means that they do not affect cells and bind directly to cellular elements; 2. are easily conjugated to monoclonal antibodies; 3. have an emission spectrum overlap as little as possible with cellular autofluorescence, which is natural fluorescence of some molecules in cells (Monici, 2005); 4. have a high cell-associated fluorescence intensity. The high fluorescence intensity enables us to distinguish positively immunofluorescence stained cells from unstained cells. The high fluorescent brightness results from fluorescence dyes with the following characteristics: 1. a high the molar absorptivity; 2. a high quantum yield; 3. the low autofluorescence; 4. a high sensitivity detector; and 5. the ability to conjugate multiple fluorescence dyes to each detecting site (Baumgarth et al., 2000).

The intensity of fluorescent dyes can be calculated by simple equations and appropriate assumptions. Fluorescent intensity could be written as (Walker, 1987)

$$I(z) = I_e(z) A \Phi L \varepsilon C \tag{1}$$

where I is the measured fluorescence intensity at time point z along the excitation beam path, Ie is the intensity of the excitation light beam at point z, A is the fraction of fluorescence light collected, Φ is the quantum efficiency, L is the length of the sampling volume along the path of the excitation beam, ε is the molar absorptivity, and C is the molar concentration of the fluorescence dye.

Fluorescent dyes	Absorbance maximum	Fluorescence emission	Molar Absorptivity $(M\text{-}cm)^{-1}$ (ε)	Quantum efficient (Φ)	Brightness (A.U.)	Brightness (*vs.* R-PE)
R-Phycoerythrin (R-PE)	490 nm 565 nm	578 nm	1,970,000	0.82	1,615,400	1
FITC	494 nm	518 nm	65,700	0.98	64,386	1/25
Propidium Iodide (PI, intercalating agent)	536 nm	716 nm	5,900	0.09	531	1/3042

Table 2. The brightness of the most common used fluorescence dyes

2.2 Two-color immunofluorescence and fluorescence dyes

Two-color immunofluorescence for two-parameter detection requires two fluorescence dyes having different emission spectra but similar excitation spectra, such as FITC and R-Phycoerythrin (R-PE). This method can be used to measure two cell populations at the same time by labeling the green florescent dye to one cell type and the red fluorescent dye to another cell type with two fluorescent detectors and a single excitation light source. To count $CD4^+$ T-cells (T-helper cells) and $CD45^+$ (leukocytes) cells which are subsets of

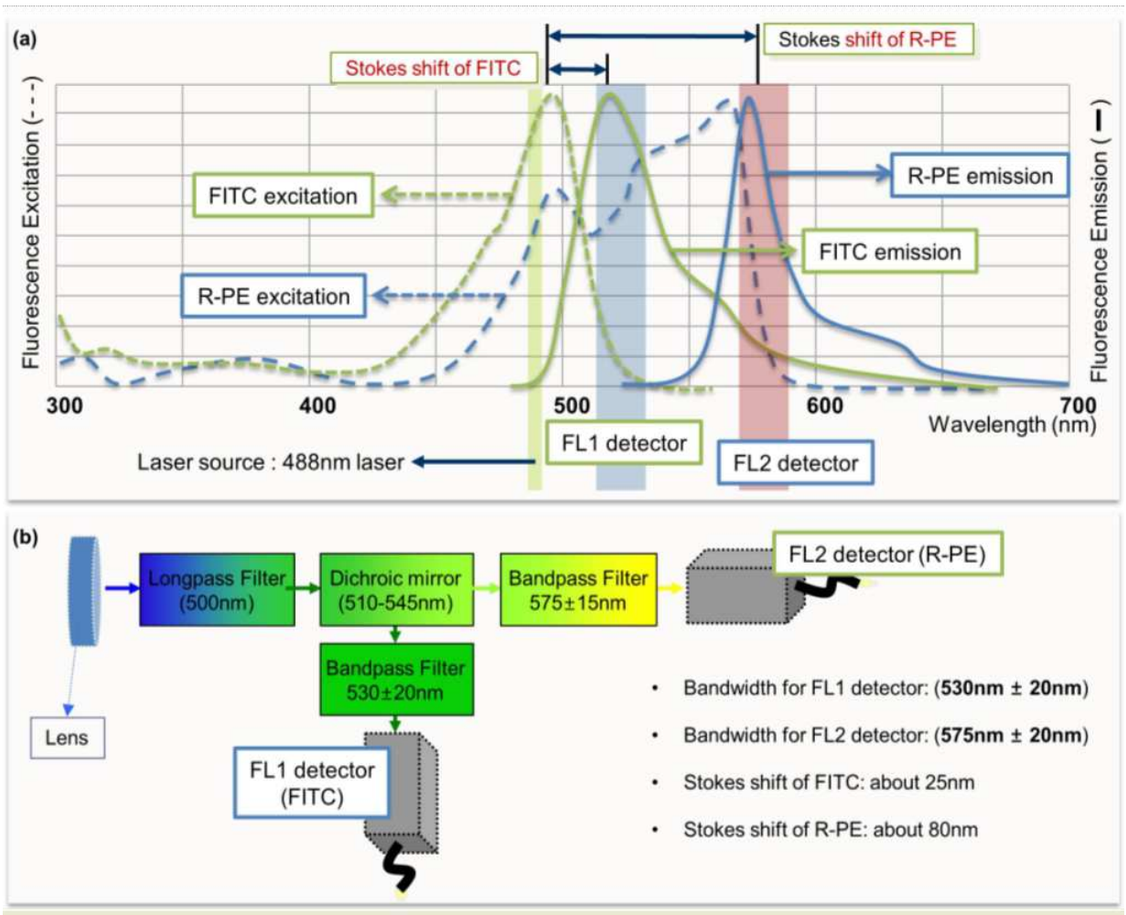


Fig. 3. (a) Excitation and emission wavelength curves of FITC and R-PE. (b) Schematic representation of an optical measurement system for two-parameter fluorescence detection. This system utilizes one laser source (488nm blue laser) and two detectors (PMTs)

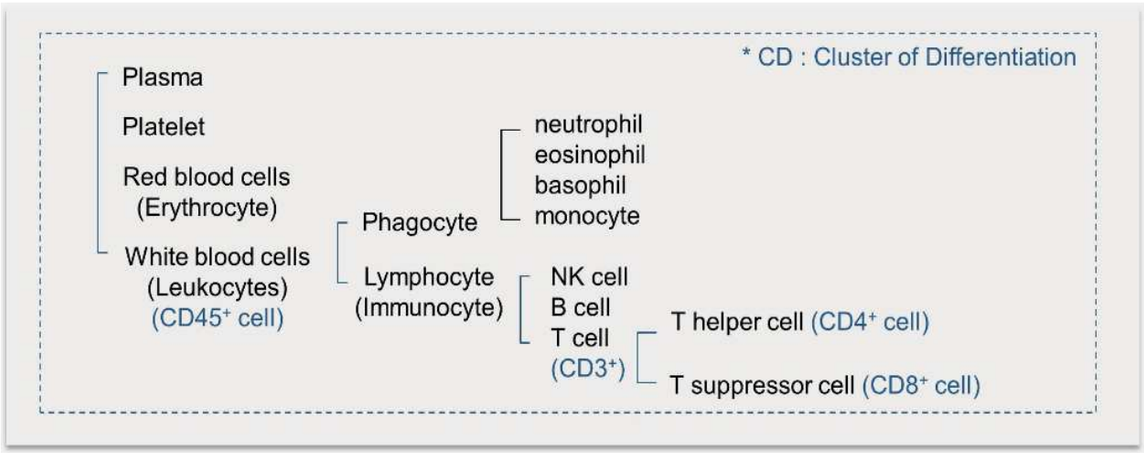


Fig. 4. A brief description of human blood subsets. T-helper cells ($CD4^+$ T-cells) are one of the most important cell types for HIV/AIDS screening because $CD4^+$ T-cells are known to be attacked by Human Immunodeficiency Virus (HIV)

leukocytes simultaneously, a mixture of FITC conjugated anti-CD45 monoclonal antibodies and R-PE conjugated anti-CD4 monoclonal antibodies are generally used. To detect additional cell types, additional fluorescence dyes with different emission wavelengths and additional detectors have to be used.

Two-color immunofluorescence utilizes a difference in the Stokes shift between two fluorescence dyes having similar excitation spectra. Therefore, we can count two different types of cells with one laser source and two PMTs. Fig. 3 shows an example of simultaneous two-parameter detection by using FITC and R-PE. The peak excitation wavelength of FITC and R-PE is 490 nm and 494 nm, respectively. 518 nm and 578 nm is the peak emission wavelength of FITC and R-PE, respectively. The optical measurement system consists of one blue laser (488 nm), one emission filter for a FITC detection (FL1, 530 ± 20 nm), and another emission filter for a R-PE detection (FL2, 575 ± 20 nm) positioned in front of each PMTs.

Fig. 5 shows an example of 2-color flow cytometry for HIV/AIDS screening. In HIV/AIDS screening, the number of $CD4^+$ T-cells in blood provides important information for antiretroviral treatment. For example, $CD4^+$ T-cell counts below 200 cells/μl require the start of antiretroviral treatment in adults (over 13 years old) (Masur et al., 2002). However, lymphocyte subsets (including $CD4^+$ T-cells) of infants and young children are higher than those of adults, therefore the ratio of $CD4^+$ T-cells to other blood cells, i.e., CD4/CD45%, CD4/CD8% or CD4/CD3%, is a more reliable indicator of HIV infection than absolute $CD4^+$ T-cell counts(Shearer et al., 2003, Organization, 2006). In general, to quantify the percentage of $CD4^+$ T-cells, two fluorescent dyes with different emission wavelengths should be assigned to each of the desired blood cell types and analyzed by a flow cytometer equipped with two PMTs. Recently, new alternative methods for affordable $CD4^+$ T-cell counting using microfluidic devices and label-free $CD4^+$ T-cell counting methods were proposed for resource-poor settings (Rodriguez et al., 2005, Cheng et al., 2007, Ateya et al., 2008).

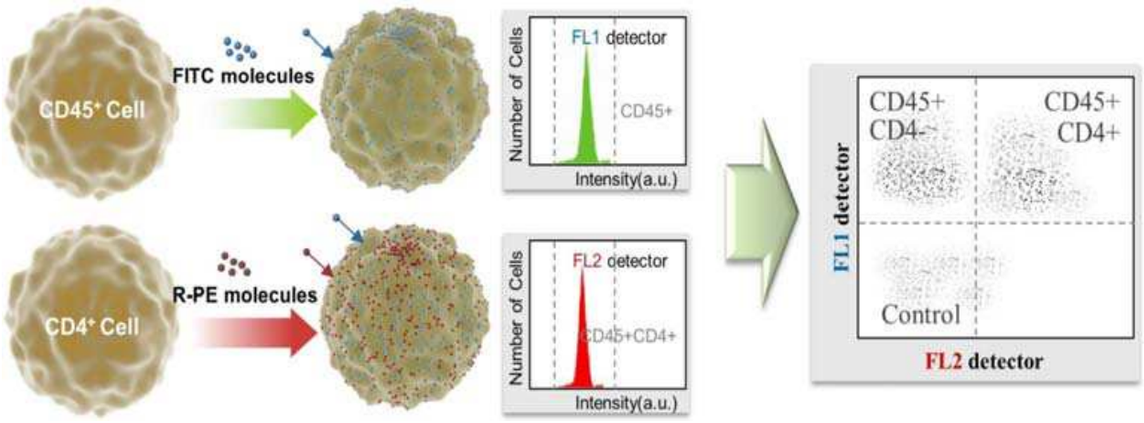


Fig. 5. An example of two-color immunofluorescence. The conventional method enables us to count two cell populations at the same time by labeling one cell type ($CD45^+$ cells) with green fluorescent dyes (FITC) and the other cell type ($CD4^+$ cells) with red fluorescent dyes (R-Phycoerythrin (R-PE)). Actually, $CD4^+$ cells are labeled with both FITC and R-PE because $CD4^+$ cells are subset of $CD45^+$ cells. HIV/AIDS screening can be performed from a simultaneous counting of $CD4^+$ T-cells and $CD45^+$ cells (Yun et al., 2010)

2.3 Multi-color immunofluorescence and fluorescence dyes

The ability to measure multi-parametric cellular information is limited by the number of fluorescence dyes that can be simultaneously measured. When designing experiments for multi-color flow cytometry that include the use of new fluorescence dye complexes, careful consideration must be given to the choice of fluorescence dyes. A desirable combination of fluorescence dyes for multi-color immunofluorescence exhibits little spectral overlap among

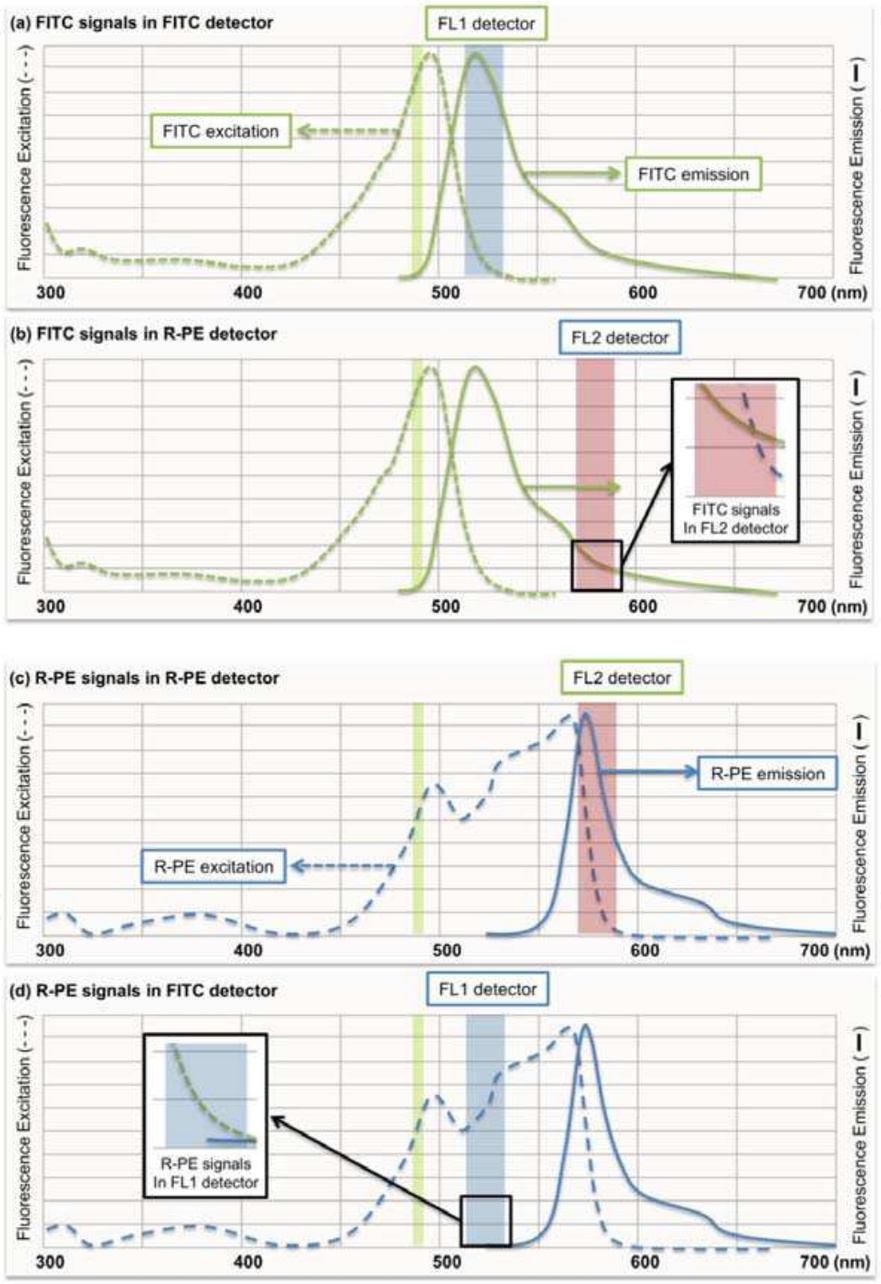


Fig. 6. The compensation problem. (a,b) FITC signals in FL1 and FL2 detectors. (c,d) R-PE signals in FL2 and FL1 detectors. FITC signals in the PE detector create most problems

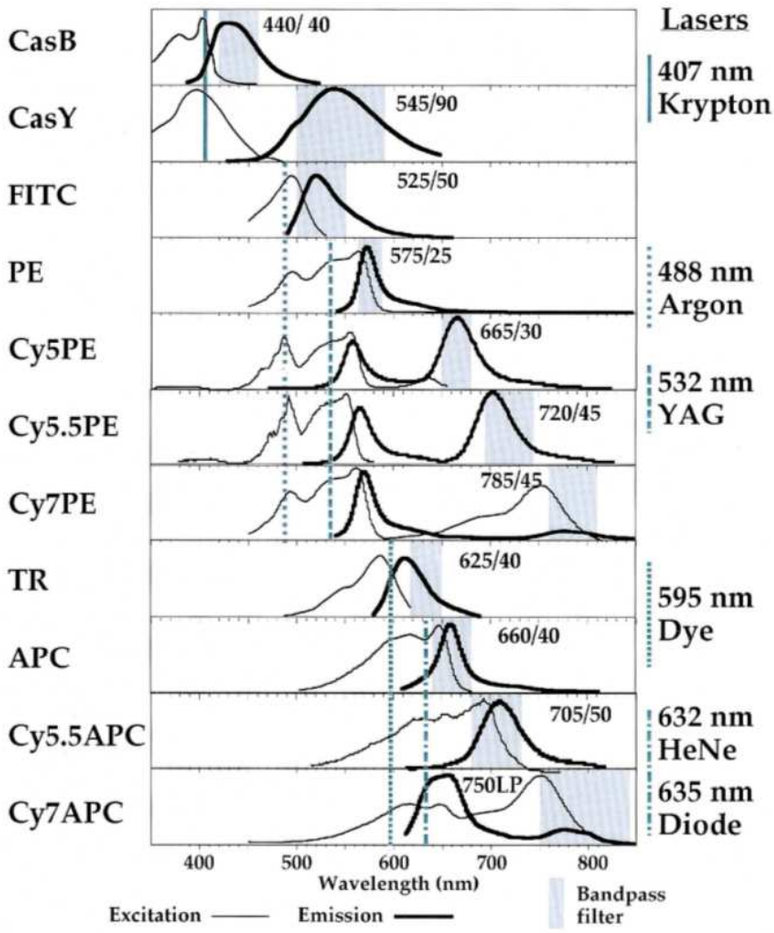


Fig. 7. An example of successful dye combination for multi-color analysis (11 colors) adapted from (Baumgarth et al., 2000). Currently, violet and green excitation light sources are provided by 405-nm violet diodes and 532-nm green solid state lasers, respectively.

each other (Baumgarth et al., 2000). The inherent overlap of emission spectra from antibody fluorescent labels makes compensation necessary. This is of particular importance when you attempt to make simultaneous immunofluorescence measurements from several cell-bound antibodies. To eliminate an error due to the overlap in the detected fluorescent signals from adjacent emission wavelengths, we should have additional compensation procedures before each flow cytometry test (Tung et al., 2004).

For flow cytometry analysis of two-parameter detection, the most common combinations of fluorescent dyes are FITC and R-PE. This is because both FITC and R-PE could be excited by a single light source such as a 488 nm blue laser but resulting in different emission spectra. However, because most fluorescent dyes do not have a sharp emission peak, the inherent overlap of emission spectra from these fluorescent labels makes compensation a necessity. In the case of FITC and R-PE in Fig. 6, spectral overlap between FITC and PE produces signals that are detected by both the FL1 and FL2 detectors. Therefore, the amount of FITC fluorescent signals being detected by the R-PE detection channel (FL2) and the amount of R-PE fluorescent signals being detected by FITC detection channel (FL1) should be

compensated and eliminated. For example, to obtain pure R-PE signals, the amount of spectral overlap can be corrected by subtracting a percentage of FITC signals from the total signal generated by the R-PE detection channel. Therefore, to make simultaneous measurements of multiple immune cell subsets, this compensation procedure should be performed before testing samples. Fig. 7 shows a successful combination of fluorescence dyes for multi-color (11-color) flow cytometry excited by three different laser lines.

3. Intensity-based multiplexed cell-counting methods

3.1 Conventional intensity-based cell-counting methods

Cell counting methods by using differences in fluorescence intensities with a single detector (a single fluorescent detection channel) have been applied to some applications such as an apoptosis measurement (Darzynkiewicz et al., 1992, Schmid et al., 2007), a bead-based absolute cell counting method (Dieye et al., 2005), a cell cycle assay based on measurement of DNA contents in a cell, or counting two specific subsets of cells having same kinds of binding sites but different number of binding sites. For example, $CD4^+$ T-cells and monocytes, which are subsets of leukocytes, have same CD4 epitopes but different averages of 47,000 and 6,500 binding sites per cell, respectively. (Mandy et al., 1997, Denny et al., 1996, Bikoue et al., 1996). In 1986, Fluorescence-intensity multiplexing methods for counting different types of cell populations using dilution of fluorophores labeled reagents with unlabeled antibodies were presented (Bradford et al., 2004, Horan et al., 1986). This study has a significant impact in multi-parametric cytometry because the method can increase the number of parameter per detector without increase of additional detectors and the compensation procedure.

3.2 Multiplexed cell-counting method using silica nanoparticles

Several types of nanoparticles such as quantum dots (QDs) (Smith et al., 2006), gold nanoparticles (Daniel et al., 2004), and dye-dope SiNPs (Yan et al., 2007, Burns et al., 2006) have been demonstrated as versatile labeling reagents for bioimaging (Sharma et al., 2006) and biosensing (Yan et al., 2007). Among them, dye-doped SiNPs provide features such as high fluorescent intensity (Ow et al., 2005), excellent photostability (Santra et al., 2001, Santra et al., 2006), and ease of surface modification for bioconjugation (Qhobosheane et al., 2001). Using dye-doped SiNPs showing 10- and 100-fold increased detection sensitivity in flow cytometry analysis compared to standard methods, Tan *et al*. have suggested a flow cytometry based cancer cell detection method when the probes have relatively weak affinities or when the receptors are expressed in low concentration on the target cell surfaces (Estevez et al., 2009). The higher brightness of dye-doped SiNPs was the main reason we adopted this nanoparticle for a proposed fluorescent intensity-based multi-cell counting method. Based on an intensity difference between fluorescent dye-doped SiNPs and conventional fluorescence dyes, the multi-parameter detection method using a single detector with flow cytometry was evaluated by carrying out simultaneous counting of $CD4^+$ T-cells and $CD45^+$ cells.

Fluorescent dyes are classified by size. Among them, small molecule fluorescence dyes such as FITC, Cy5, and Alexa dyes could be doped to directly nanoparticles to obtain brighter fluorescent dyes complexes while maintaining a same excitation and emission spectra. On the other hand, fluorescent proteins such as R-PE, allophycocyanin (APC) cannot be directly

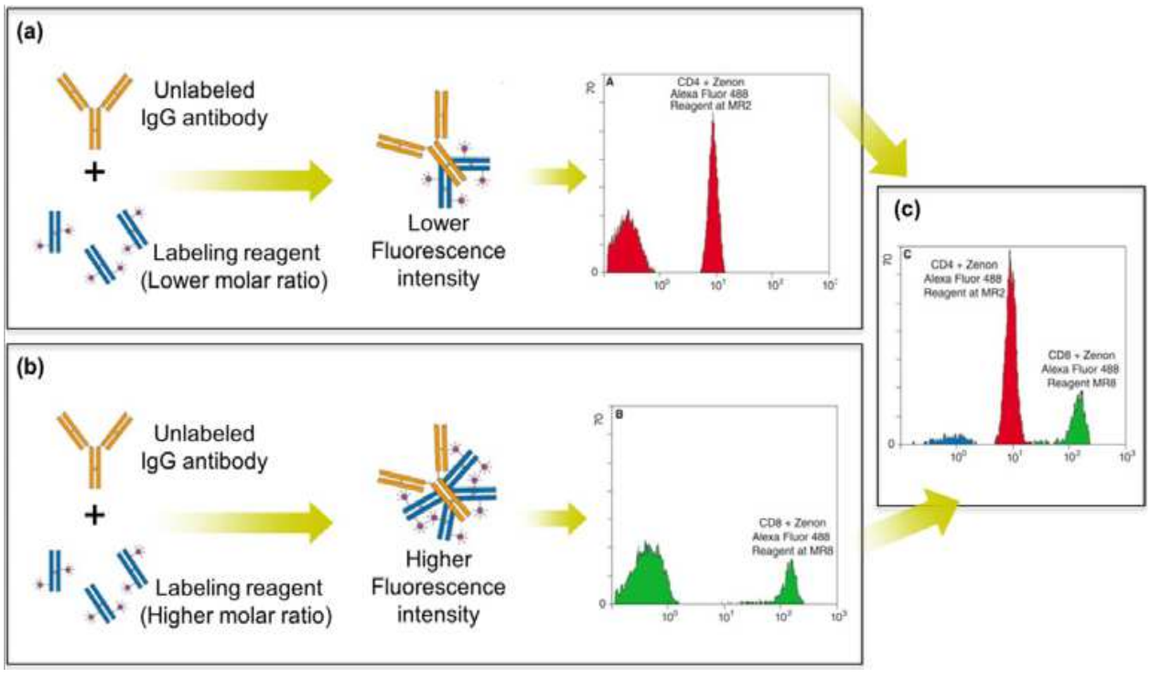


Fig. 8. Fluorescence-intensity multiplexing analysis by varying the labeling reagent (fluorescence dyes)-to antibody molar ratio (Bradford et al., 2004). This immunolabeling technology allows for multiple antigen detection per detection channel using a single fluorophore. (a) Labeling scheme for lower fluorescence intensity. Histogram shows $CD4^+$ T-cells labeling with a complex of CD4 antibodies and reagents having lower fluorescence intensity (a molar ratio of a labeling reagent to a primary antibody is 2). (b) Labeling scheme for higher fluorescence intensity. Histogram shows $CD8^+$ T-cells labeling with a complex of CD8 antibodies and fluorescence reagents having higher intensity (a molar ratio of a labeling reagent to a primary antibody is 8). (c) Histogram of simultaneous counting of $CD4^+$ cells and $CD8^+$ cells by a single detection channel

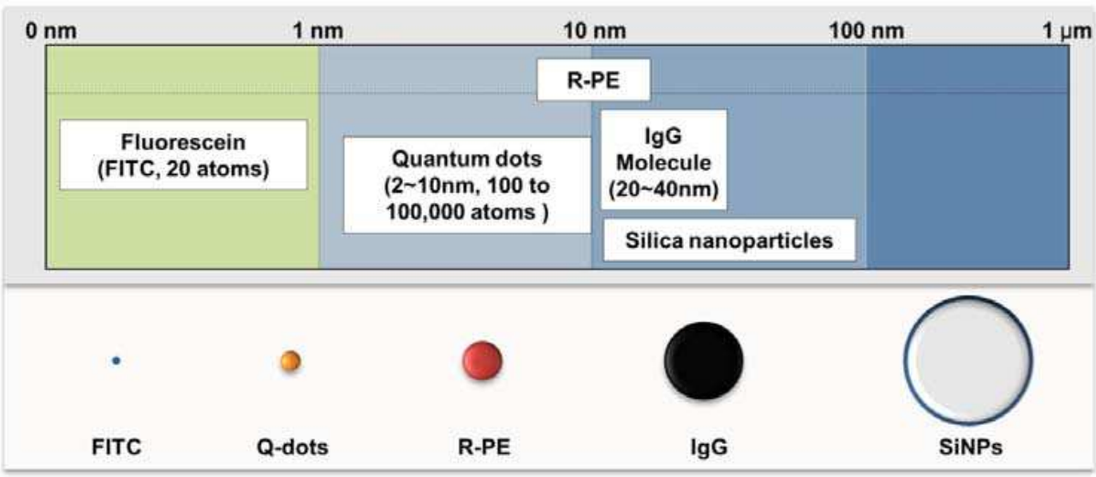


Fig. 9. The size of materials, including several types of fluorophores, immunoglobulin G (IgG), quantum dots, and silica nanoparticles

doped to nanoparticles because fluorescent proteins are much larger than small fluorophores relatively. In order to apply fluorescent proteins to intensity-based cell counting, fluorescent proteins should be used with fluorophores doped silica nanoparticles which have similar excitation and emission wavelengths with the fluorescent proteins.

The intensity of those fluorescent dye complexes can be calculated by simple equations and appropriate assumptions. From that calculation we can obtain a feasible combination of fluorophores doped nanoparticles and fluorescent proteins for intensity-based differential counting. To use fluorescent proteins in intensity based cell-counting, ideally, small molecule fluorophores having identical excitation and emission wavelengths as the fluorescent proteins itself need to be tagged to the SiNPs. Because there was no readily available combination of fluorophores and fluorescent proteins with same emission wavelengths, fluorophores which have adjacent excitation and emission wavelengths with fluorescent proteins was selected. The intensity of fluorescent dye-doped SiNPs can be calculated as following.

$$I(z) = n \times I_e(z) A \Phi L \varepsilon C \tag{2}$$

where n is the number of fluorophores on a single silica nanoparticle. For example, the number of FITC molecules on a single nanoparticle (n) can be calculated theoretically as following Table 2. The majority of fluorescent dyes have a nonspherical shape. Fluorescein (FITC) is also a nonspherical solute with sizes of 0.47, 0.81, and 1.09 nm in different directions (Cvetkovic et al., 2005). Therefore, the size of fluorescent dye should be determined by using the appreciate method.

Method	size	The number of dyes per SiNP	Brightness (vs. FITC-IgG)
The stokes radius (Deen, 1987)	0.44 nm	40,568	5,795
The density of dyes (1kDa/1nm3)	0.9 nm	9696	1,385
The equivalent spherical diameter (Jennings et al., 1988, Cvetkovic et al., 2005)	0.7 nm	16,028	2,289
The maximum size	1.1 nm	6490	927

Table 3. Theoretical calculation of brightness of FITC-doped SiNPs

Table 3 shows the sizes (ranging from 0.44 nm to 1.1 nm) of a single FITC molecule with different methods. Therefore, the number of FITC molecule per a single nanoparticle is theoretically from 6490 to 40,568 and the relative intensity of FITC-doped SiNPs in FITC conjugated IgG is from 927 to 2,289. This theoretical value of the intensity deference is higher than experimental results from previous studies (Lian et al., 2004, Ow et al., 2005, Estevez et al., 2009, Yun et al., 2010). These results demonstrated dye-doped SiNPs is 10-100 times brighter than their constituent fluorophore. The reason for this relatively low intensity from the theoretical calculation is because fluorescent dyes were lost during the synthesis of dyes-doped SiNPs or photobleached (Santra et al., 2006). When considering these factors, the above equation of fluorescence intensity (2) is written as follows.

$$I_{SiNPs} = \left[n \times I_e(z) A\Phi L\varepsilon C\right] \times P \times L \tag{3}$$

Where P is the factor of photobleaching ranging from 0 to 1 and L is the factor of the fraction of remaining dyes ranging from 0 to 1. Accordingly, the intensity of small fluorophores doped SiNPs relative to a fluorescent protein can be defined as

$$I_{RELATIVE} = \frac{\left[I_e(z) A\Phi L\varepsilon C\right]_{PROTEIN}}{\left[n \times I_e(z) A\Phi L\varepsilon C \times P \times L\right]_{SiNPs}} \tag{4}$$

If $I_{relative} \geq 100$ or $I_{relative} \leq 0.01$, the intensity based cell-counting method using the intensity difference between small fluorophore doped SiNPs and fluorescent protein can be applied. For example, when using Propidium Iodide (PI, ε=5900/*M*/*Cm* and Φ=0.09) in combination with R-PE, Equation 4 could be transformed and simplified as 15,000/nPL approximately. Therefore, at conditions of no photobleaching (P=1) and no loss during the synthesis (L=1), less than 150 or more than 1,500,000 PI molecules need to dope SiNPs to analyze the two parameters using R-PE conjugated antibodies and PI-doped SiNPs.

Fig. 10 shows a concept of simultaneous counting of two subsets of leukocytes by using a combination of FITC-doped silica nanoparticles and FITC molecules. Although this study showed good correlation between the proposed method and a conventional method (R = 0.936, R^2 = 0.876), regression analysis from these results is not sufficient yet for the developed method to replace the conventional method in clinical setting. Some technical issues, such as nonspecific binding of silica nanoparticles, should be resolved.

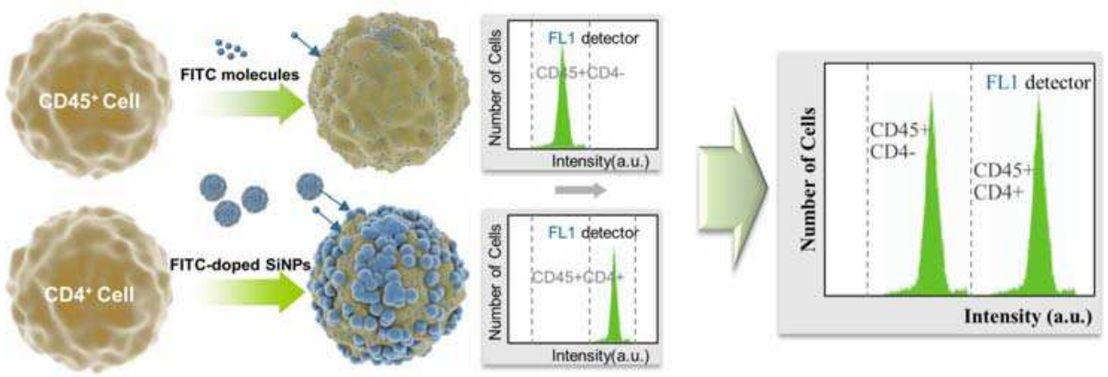


Fig. 10. An example of multiplexed cell counting using silica nanoparticles. This method utilized a dye combination of FITC and FITC-doped SiNPs instead of R-PE (Yun et al., 2010). Actually, $CD4^+$ cells are labeled with both FITC and FITC-doped SiNPs because $CD4^+$ cells are subset of $CD45^+$ cells

3.3 Multiplexed cell-counting method using quantum dots

Instead of the method by using much brighter fluorescence dyes such as FITC-doped silica nanoparticles, a method by using much darker dyes than general fluorophores can be applied to the multiplexed cell counting. Fig. 11 shows this counting concept. The proposed method also enables simultaneous counting of two subsets of leukocytes using a single detector by using quantum dots 605 (QDs 605) instead of FITC dyes in the conventional method. A combination of Q-dots 605 and R-Phycoerythrin (R-PE) can be used for making a

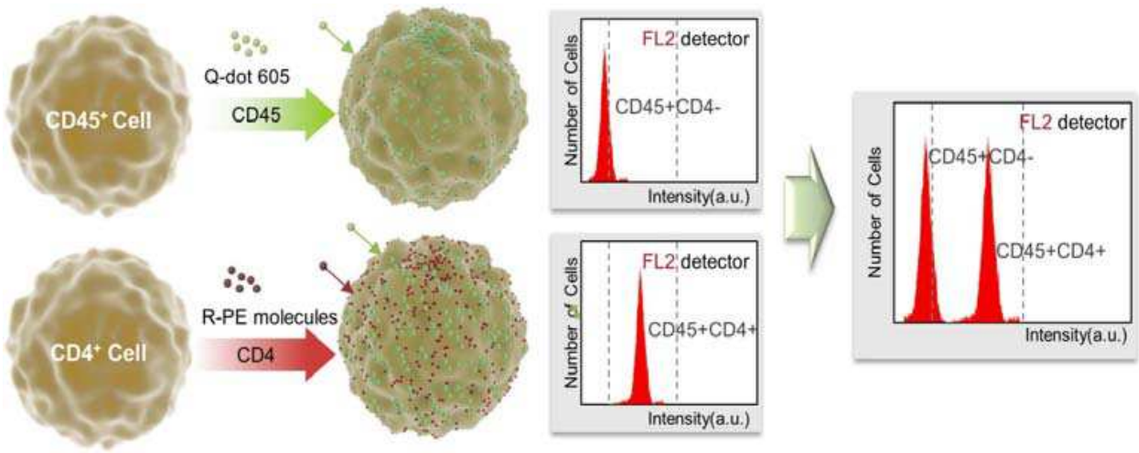


Fig. 11. An example of multiplexed cell counting using quantum dots. By using a complex of Q-dot 605 conjugated CD45+ cells and R-PE conjugated CD4+ T-cells with a specific emission filter (from 564 to 606 nm), we can simultaneously count two different cell types (CD45+ and CD4+ T-cells) in a single fluorescent channel. Actually, CD4+ cells are labeled with both Q-dot 605 and R-PE because CD4+ cells are subset of CD45+ cells

similar effect in the multiplexed counting method with dye-doped silica nanoparticles. Q-dots 605 and R-PE have similar fluorescence intensity with a wide band width filter. However, by using a specific emission filter (from 564 to 606 nm), Q-dots 605 conjugated antibodies were detected as 10-100 times darker than R-PE conjugated antibodies.

4. Conclusion

Conventional flow cytometry requires multiple detectors for simultaneous identification of multiple subsets of immune cells because this method measures a single fluorescence dyes conjugated antibody per detector (PMTs). The ultimate goal of multiplexed cell-counting methods is to increase detectable parameters per fluorescence channel. These methods enable us to simultaneously measure multiple types of samples using a single detector without a troublesome fluorescence compensation procedure. In order to use the intensity-based counting in various fluorescent fields, this chapter suggests feasible combinations of fluorescence dyes and theoretical analysis to quantify an intensity difference between combinations. The combinations are classified into three groups; 1) fluorophores and fluorophore-doped silica nanoparticles which have same excitation and emission wavelengths; 2) fluorescent proteins and fluorophore-doped silica nanoparticles which have similar excitation and emission wavelengths; 3) combinations of fluorescence dyes such as quantum dots 605 and R-PE, which have different excitation and emission wavelengths. Multiplexed cell-counting methods in this chapter can be applied to both high performance flow cytometers for measurement of multiple parameters on cells and inexpensive portable flow cytometers. By using the ability of these intensity-based cell counting methods, conventional flow cytometers can detect more parameters without increase of detectors and portable flow cytometers can minimize the number of detectors. This study can be the building block for a more powerful and truly portable flow cytometer for various clinical cytometry applications.

5. Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0005219)

6. References

Ateya, D. A., Erickson, J. S., Howell, P. B., Hilliard, L. R., Golden, J. P. & Ligler, F. S. 2008. The good, the bad, and the tiny: a review of microflow cytometry. *Analytical and Bioanalytical Chemistry,* Vol. 391, No. 5, pp. 1485-1498,

Baumgarth, N. & Roederer, M. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. *Journal of immunological methods,* Vol. 243, No. 1-2, pp. 77-97,

Bikoue, A., George, F., Poncelet, P., Mutin, M., Janossy, G. & Sampol, J. 1996. Quantitative analysis of leukocyte membrane antigen expression: normal adult values. *Cytometry Part B: Clinical Cytometry,* Vol. 26, No. 2, pp. 137-147,

Bonetta, L. 2005. Flow cytometry smaller and better. *Nature Methods,* Vol. 2, No. 10, pp. 785-795, 1548-7091

Bradford, J., Buller, G., Suter, M., Ignatius, M., Beechem, J., Probes, M. & Eugene, O. 2004. Fluorescence-intensity multiplexing: simultaneous seven-marker, two-color immunophenotyping using flow cytometry. *Cytometry Part A,* Vol. 61A, No. 2, pp. 142-152,

Brando, B., Barnett, D., Janossy, G., Mandy, F., Autran, B., Rothe, G., Scarpati, B., D'avanzo, G., D'hautcourt, J.-L., Lenkei, R., Schmitz, G., Kunkl, A., Chianese, R., Papa, S. & Gratama, J. W. 2000. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. *Cytometry,* Vol. 42, No. 6, pp. 327-346, 1552-4957

Burns, A., Ow, H. & Wiesner, U. 2006. Fluorescent core-shell silica nanoparticles: towards "Lab on a Particle" architectures for nanobiotechnology. *Chemical Society Reviews,* Vol. 35, No. 11, pp. 1028-1042,

Cheng, X., Irimia, D., Dixon, M., Sekine, K., Demirci, U., Zamir, L., Tompkins, R. G., Rodriguez, W. & Toner, M. 2007. A microfluidic device for practical label-free CD4+ T cell counting of HIV-infected subjects. *Lab on a Chip,* Vol. 7, No. 2, pp. 170-178,

Cohen, J. 2004. Monitoring treatment: At what cost? *Science,* Vol. 304, No. 5679, pp. 1936-1936, 0036-8075

Coons, A. H., Creech, H. J. & Jones, R. N. 1941. Immunological properties of an antibody containing a fluorescent group. *Experimental Biology and Medicine,* Vol. 47, No. 2, pp. 200, 1535-3702

Cottingham, K. 2005. Incredible shrinking flow cytometers. *Analytical Chemistry,* Vol. 77, No. 3, pp. 73a-76a,

Coulter, W. H. 1953. *Means for counting particles suspended in a fluid*. United States patent application 2656508.

Crosland-Taylor, P. 1953. A device for counting small particles suspended in a fluid through a tube. *Nature,* Vol., No. 171, pp. 37-38,

Cvetkovic, A., Picioreanu, C., Straathof, A., Krishna, R. & Van Der Wielen, L. 2005. Relation between pore sizes of protein crystals and anisotropic solute diffusivities. *J. Am. Chem. Soc,* Vol. 127, No. 3, pp. 875-879,

Daniel, M. & Astruc, D. 2004. Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. *Chem. Rev,* Vol. 104, No. pp. 293-346,

Darzynkiewicz, Z., Bedner, E., Li, X., Gorczyca, W. & Melamed, M. R. 1999. Laser-Scanning Cytometry: A New Instrumentation with Many Applications. *Experimental Cell Research,* Vol. 249, No. 1, pp. 1-12,

Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P. & Traganos, F. 1992. Features of apoptotic cells measured by flow cytometry. *Cytometry,* Vol. 13, No. 8, pp. 795-808,

Deen, W. 1987. Hindered transport of large molecules in liquid filled pores. *AIChE Journal,* Vol. 33, No. 9, pp. 1409-1425, 1547-5905

Denny, T., Stein, D., Mui, T., Scolpino, A., Holland, B. & Endowment, F. 1996. Quantitative determination of surface antibody binding capacities of immune subsets present in peripheral blood of healthy adult donors. *Cytometry Part B: Clinical Cytometry,* Vol. 26, No. 4, pp. 265-274,

Dieye, T. N., Vereecken, C., Diallo, A. A., Ondoa, P., Diaw, P. A., Camara, M., Karam, F., Mboup, S. & Kestens, L. 2005. Absolute CD4 T-cell counting in resource-poor settings: direct volumetric measurements versus bead-based clinical flow cytometry instruments. *JAIDS Journal of Acquired Immune Deficiency Syndromes,* Vol. 39, No. 1, pp. 32, 1525-4135

Estevez, M., O'donoghue, M., Chen, X. & Tan, W. 2009. Highly fluorescent dye-doped silica nanoparticles increase flow cytometry sensitivity for cancer cell monitoring. *Nano Research,* Vol. 2, No. 6, pp. 448-461,

Fulwyler, M. 1965. Electronic separation of biological cells by volume. *Science,* Vol. 150, No. 3698, pp. 910,

Glencross, D., Scott, L., Jani, I., Barnett, D. & Janossy, G. 2002. CD45-assisted PanLeucogating for accurate, cost-effective dual-platform CD4+ T-cell enumeration. *Cytometry Part B: Clinical Cytometry,* Vol. 50, No. 2, pp. 69-77,

Gucker Jr, F., O'konski, C., Pickard, H. & Pitts Jr, J. 1947. A Photoelectronic Counter for Colloidal Particles1. *Journal of the American Chemical Society,* Vol. 69, No. 10, pp. 2422-2431, 0002-7863

Herzenberg, L. & Sweet, R. 1976. Fluorescence-activated cell sorting. *Sci Am,* Vol. 234, No. 3, pp. 108-117,

Horan, P., Slezak, S. & Poste, G. 1986. Improved flow cytometric analysis of leukocyte subsets: simultaneous identification of five cell subsets using two-color immunofluorescence. *Proceedings of the National Academy of Sciences,* Vol. 83, No. 21, pp. 8361-8365,

Houwen, B. 2001. The Differential Cell Count. *Laboratory Hematology,* Vol. 7, No. 2, pp. 89-100,

Janossy, G., Jani, I. & Gohde, W. 2000. Affordable CD4+ T-cell counts on'single-platform'flow cytometers I. Primary CD4 gating. *British Journal of Haematology,* Vol. 111, No. 4, pp. 1198-1208,

Janossy, G., Jani, I. V. & Brando, B. 2003. New trends in affordable CD4+ T-cell enumeration by flow cytometry in HIV/AIDS. *Clinical and Applied Immunology Reviews,* Vol. 4, No. 2, pp. 91-107,

Jennings, B. & Parslow, K. 1988. Particle size measurement: the equivalent spherical diameter. *Proceedings of the Royal Society of London. A. Mathematical and Physical Sciences,* Vol. 419, No. 1856, pp. 137, 1364-5021

Kapoor, V., Subach, F. V., Kozlov, V. G., Grudinin, A., Verkhusha, V. V. & Telford, W. G. 2007. New lasers for flow cytometry: filling the gaps. *Nature Methods,* Vol. 4, No. 9, pp. 678-679,

Laerum, O. D. & Farsund, T. 1981. Clinical application of flow cytometry: a review. *Cytometry,* Vol. 2, No. 1, pp. 1-13,

Ledbetter, J. A., Rouse, R. V., Micklem, H. S. & Herzenberg, L. 1980. T cell subsets defined by expression of Lyt-1, 2, 3 and Thy-1 antigens. Two-parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. *The Journal of experimental medicine,* Vol. 152, No. 2, pp. 280, 0022-1007

Lee, W., Kim, Y., Chung, B., Demirci, U. & Khademhosseini, A. 2010. Nano/Microfluidics for diagnosis of infectious diseases in developing countries. *Advanced Drug Delivery Reviews,* Vol. 62, No. pp. 449-457,

Lian, W., Litherland, S., Badrane, H., Tan, W., Wu, D., Baker, H., Gulig, P., Lim, D. & Jin, S. 2004. Ultrasensitive detection of biomolecules with fluorescent dye-doped nanoparticles. *Analytical Biochemistry,* Vol. 334, No. 1, pp. 135-144,

Loken, M., Parks, D. & Herzenberg, L. 1977. Two-color immunofluorescence using a fluorescence-activated cell sorter. *J Histochem Cytochem,* Vol. 25, No. 7, pp. 899-907,

Mandy, F. F., Bergeron, M. & Minkus, T. 1997. Evolution of Leukocyte Immunophenotyping as Influenced by the HIV/AIDS Pandemic: A Short History of the Development of Gating Strategies for CD4 T-Cell Enumeration. *Cytometry (Communications in Clinical Cytometry),* Vol. 30, No. pp. 157-165,

Masur, P. H., Kaplan, J. E. & Holmes, K. K. 2002. Guidelines for Preventing Opportunistic Infections among HIV-Infected Persons-2002: Recommendations of the US Public Health Service and the Infectious Diseases Society of America*. *Annals of Internal Medicine,* Vol. 137, No. 5 Part 2, pp. 435-478,

Moldavan, A. 1934. Photo-electric technique for the counting of microscopical cells. *Science,* Vol. 80, No. pp. 188-189, 0036-8075

Monici, M. 2005. Cell and tissue autofluorescence research and diagnostic applications. *Biotechnology Annual Review,* Vol. 11, No. pp. 227-256, 1387-2656

Murphy, R. & Chused, T. 1984. A proposal for a flow cytometric data file standard. *Cytometry,* Vol. 5, No. 5, pp. 553-555, 1097-0320

Organization, W. H. 2006. HIV/AIDS Programme, Antiretroviral Therapy of HIV Infection in Infants and Children in Resource-Limited Settings: Towards Universal Access - Recommendations for a Public Health Approach. World Health Organization.

Ow, H., Larson, D. R., Srivastava, M., Baird, B. A., Webb, W. W. & Wiesner, U. 2005. Bright and stable core-shell fluorescent silica nanoparticles. *Nano Lett,* Vol. 5, No. 1, pp. 113-117,

Parks, D., Hardy, R. & Herzenberg, L. 1984. Three color immunofluorescence analysis of mouse B lymphocyte subpopulations. *Cytometry,* Vol. 5, No. 2, pp. 159-168, 1097-0320

Perfetto, S. P., Chattopadhyay, P. K. & Roederer, M. 2004. Seventeen-Colour Flow Cytometry: Unravelling The Immune System. *Nature Reviews Immunology,* Vol. 4, No. pp. 648-655,

Qhobosheane, M., Santra, S., Zhang, P. & Tan, W. 2001. Biochemically functionalized silica nanoparticles. *The Analyst,* Vol. 126, No. 8, pp. 1274-1278,

Rodriguez, W. R., Christodoulides, N., Floriano, P. N., Graham, S., Mohanty, S., Dixon, M., Hsiang, M., Peter, T., Zavahir, S. & Thior, I. 2005. A Microchip CD4 Counting Method for HIV Monitoring in Resource-Poor Settings. *PLoS Medicine,* Vol. 2, No. 7, pp. e182,

Santra, S., Liesenfeld, B., Bertolino, C., Dutta, D., Cao, Z., Tan, W., Moudgil, B. M. & Mericle, R. A. 2006. Fluorescence lifetime measurements to determine the core?shell nanostructure of FITC-doped silica nanoparticles: An optical approach to evaluate nanoparticle photostability. *Journal of Luminescence,* Vol. 117, No. 1, pp. 75-82,

Santra, S., Zhang, P., Wang, K., Tapec, R. & Tan, W. 2001. Conjugation of biomolecules with luminophore-doped silica nanoparticles for photostable biomarkers. *Analytical Chemistry,* Vol. 73, No. 20, pp. 4988-93,

Schmid, I., Uittenbogaart, C. & Jamieson, B. D. 2007. Live-cell assay for detection of apoptosis by dual-laser flow cytometry using Hoechst 33342 and 7-amino-actinomycin D. *Nature Protocols,* Vol. 2, No. 1, pp. 187-190,

Shapiro, H. M. 1983. Multistation multiparameter flow cytometry: a critical review and rationale. *Cytometry,* Vol. 3, No. 4, pp. 227-43,

Sharma, P., Brown, S., Walter, G., Santra, S. & Moudgil, B. 2006. Nanoparticles for bioimaging. *Advances in colloid and interface science,* Vol. 123, No. pp. 471-485,

Shearer, W. T., Rosenblatt, H. M., Gelman, R. S., Oyomopito, R., Plaeger, S., Stiehm, E. R., Wara, D. W., Douglas, S. D., Luzuriaga, K. & Mcfarland, E. J. 2003. Lymphocyte subsets in healthy children from birth through 18 years of age The pediatric AIDS clinical trials group P1009 study. *The Journal of Allergy and Clinical Immunology,* Vol. 112, No. 5, pp. 973-980,

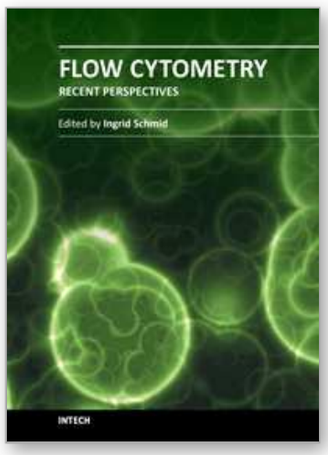
Smith, A., Dave, S., Nie, S., True, L. & Gao, X. 2006. Multicolor quantum dots for molecular diagnostics of cancer. *Expert Review of Molecular Diagnostics,* Vol. 6, No. 2, pp. 231-244,

Tung, J. W., Parks, D. R., Moore, W. A. & Herzenberg, L. A. 2004. New approaches to fluorescence compensation and visualization of FACS data. *Clinical Immunology,* Vol. 110, No. 3, pp. 277-283,

Walker, D. A. 1987. A fluorescence technique for measurement of concentration in mixing liquids. *Journal of Physics E: Scientific Instruments,* Vol. 20, No. pp. 217-224,

Yan, J., Estevez, M., Smith, J., Wang, K., He, X., Wang, L. & Tan, W. 2007. Dye-doped nanoparticles for bioanalysis. *Nano Today,* Vol. 2, No. 3, pp. 44-50,

Yun, H., Bang, H., Min, J., Chung, C., Chang, J. K. & Han, D. C. 2010. Simultaneous counting of two subsets of leukocytes using fluorescent silica nanoparticles in a sheathless microchip flow cytometer. *Lab on a Chip,* Vol. 10, No. pp. 3243-3254,



Flow Cytometry - Recent Perspectives

Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0626-5
Hard cover, 500 pages
Publisher InTech
Published online 13, June, 2012
Published in print edition June, 2012

"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Hoyoung Yun, Won Gu Lee and Hyunwoo Bang (2012). Multiplexed Cell-Counting Methods by Using Functional Nanoparticles and Quantum Dots, Flow Cytometry - Recent Perspectives, M.Sc. Ingrid Schmid (Ed.), ISBN: 978-953-51-0626-5, InTech, Available from: http://www.intechopen.com/books/flow-cytometry-recent-perspectives/multiplexed-cell-counting-methods-by-using-functional-nanoparticles-and-quantum-dots

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen