The development of multicolor panels with Quantum Dots-conjugated antibodies for diagnostics of Chronic Lymphoid Leukemia (CLL) by conventional flow cytometer

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ABSTRACT

The standard flow cytometry diagnostics approach (3 colors) requires 10-15 tubes per patient and does not allow identification of small populations of tumor cells. Quantum dots (QDots) offer new possibilities in polychromatic clinical cytometry resolving organic and tandem dyes issues with stability and donor bleed-through. We used combinations of beads stained with different antibody conjugates with QDots and standard fluorochromes to calculate signal to noise ratio and stain index for QD605, QD655 and QD705-conjugated antibodies alone and included in our CLL panels. We developed 8-color panel (CD3/CD5/CD19/CD23/CD38/CD10/CD45/ Sytox Blue viability dye) panel incorporating QD605, QD655 and QD705 direct antibody conjugates (Invitrogen) for differential diagnostics of CLL. Using this panel we were able to define the population of CLL cells that was as small as 1% of the overall PBMC population – it was determined in dilution experiments (adding mixture of ~0.2% CLL to the normal blood).

Keywords: quantum dots, antibodies, chronic lymphoid leukemia, flow cytometry.

1 INTRODUCTION

Diagnostics of leukemia and lymphomas by flow cytometry emerged in the 90's when several consensus panels had been introduced. However, since that time diagnostics has been based on three color cytometry and for each patient up to 10-15 tubes would be used. The standard flow diagnostics approach does not allow for identification of small populations of tumor cells. The use of a 4-5-color diagnostic panel significantly increased the ability to identify small populations, however it is not routine [1-3]. The major obstacles for development of multicolor diagnostics are: 1) problem of multicolor compensation; 2) problem of different brightness of existing fluorophores. Since tumor cells often express diagnostic markers at the level below their normal counterparts, strong fluorescent signal from each conjugated antibody is essential. Fluorescent semiconductor nanocrystals (quantum dots, QDots) offer new possibilities in polychromatic cytometry [4]. In contrast with conventional organic fluorophores - QDots are photostable, have a broad absorption and narrow emissions spectra. In this work we have used several commercially available QDots-antibody conjugates (Invitrogen, Carlsbad, USA) as a first step in the development of multicolor panels for CLL diagnostics.

2 METHODS

Reagents. Anti-human CD3, CD5, CD19, CD23, CD38 CD10, CD45 conjugates with conventional and tandem fluorophores were acquired from Invitrogen (Carlsbad, USA), Ebioscience (San Diego, USA) and BD Biosciences (San Jose, USA). Anti-human CD3-QDot 605, CD38-QDot 605, CD19-QDot 655, CD3 QDot705, CD4-QDot 705 were obtained from Invitrogen (Carlsbad, CA). We used combinations of compensation beads from BD Biosciences (San Jose, CA) stained with different antibodies conjugated with QDots or standard fluorochromes to calculate spectral compensation, signal to noise ratio, and stain ratio for QD605, QD655 and QD705 included in our CLL panels. Stain Index (SI) was calculated as (mean $_{positive}$ – mean $_{background}$ / 2 x SD $_{background}$).

Human blood from CLL patients and normal controls was collected, peripheral blood mononuclear cells (PBMC) were obtained by ficoll-centrifugation, and stained with a combination of various 8-color panels or FMO ("minus one"controls).

Fluorescent staining of bead combinations, peripheral blood leukocytes from CLL patients, and normal controls were acquired and analysed with FACSCanto2 cytometer (BDBiosciences, San Jose, CA) equipped with a standard combination of three lasers (405, 488 and 635 nm excitation), 10-parameters detection capabilities (4 blue fluorescence channels, two red fluorescence channels, two violet fluorescence channels and forward and side light scatter from the 488 nm laser) and a set of optical filters from BD Biosciences for FACSCanto2. We analyzed 100,000 PBMC per sample with DiVa software or software for off-line analysis FlowJo (Treestar, Ashland, USA).

3 RESULTS

Excitation (nm)	Emission Filter
405	450/50
405	510/50
488	530/30

488	585/42
488	670LP
488	780/60
635	660/20
635	780/60 or 720/40

Table 1: Configuration of FACSCanto2: laser excitation wavelengths and emission filters.

The excitation wavelengths and emission filters for the FACSCanto2 cytometer used in this study are shown in Table 1. Though optimal excitation for QDots is obtained with 355 nm or 405 nm lasers, the QDots can be suboptimally excited with 488 nm or 633 nm common lasers.

Channel	Compensation vs QDot 655
FITC	0.02%
PE	0.6%
PE-Cy7	0.28%
PERCP	117.44%
PE-Cy7	0.28%
2nd red laser channel	0.11%
AmCyan	0.19%

Table 2. Example of compensation values obtained for tested QDots on conventional flow cytometer FACSCanto2.

The spillover of other fluorochromes into the QDot 655 channel was minimal (between 0.02% and 0.6%). The greatest spillover of QDot 655 in other channels was on PERCP channel (detected with 670LP filter). There was practically no spillover to violet fluorescence channels due to standard optical configuration of cytometer out of emission range for QDots 605, 655 and 705 (standard set of filters supplied by BD Biosciences). The spillover from QD705 was relatively high in PE-Cy7 and APC-Cy7 channels. Compensation values are dependent from used photomultipliers (PMTs) voltages, and so practically impossible to define compensation or spillover of fluorophores by simple percentage.

Fluorophore	CD3-QD605	CD19-QD655	CD4-QD705	CD3-PE	CD4-PE
Negative signal	13	48	23	8	9
Positive signal	2234	3547	11372	20805	15598

Table 3. Relative brightness of antibodies directly conjugated with different QDots or Phycoerythrin (surface staining).

In the suggested 8-color multicolor cocktail (CD3 (orCD4)/CD5/CD19/CD23/CD38/CD10/CD45/Sytox Blue viability dye) relative brightness of tested QDots is less comparing with phycoerythrin-conjugated antibodies, but comparable or higher than FITC or Pacific Blue. An SI comparison also demonstrated the superior resolution of the some QDots reagents, where CD4-QD705 antibody has an SI 4.2 times that of CD4-FITC.

Using this 8-color cocktail we were able to define the population of CLL cells that was as small as 1% of the overall PBMC population – which was determined in dilution experiments (adding mixture of ~0.2% CLL to the normal blood - data not shown).

4 DISCUSSION

In this study we have used some commercially available QDots-conjugated antibodies with a view of development of rational basis for building multicolor diagnostic panels for conventional flow cytometer using longer wavelength lasers for QDots excitation.

QDots nanoparticles have inherently broad excitation spectra, and the efficiency of excitation varies with the wavelength of the laser line. QDots are very bright fluorophores when excited with UV or 405 nm lasers. In this study Qdots were efficiently excited with longer wavelengths lasers, which are still standard for many laboratory analyzers, including FACSCalibur (488 and 635 nm) from BD Biosciences (San Jose, USA), Cytomics FC500 (488 nm and 635 nm) from Beckman Coulter (Miami, USA) and others.

However, in the case of excitation with 488 and 633 nm lasers as a part of multicolor panel with organic and tandem fluorochromes QDots may require significant compensation between detectors due to their bleeding through in some channels.

This study can be helpful for the development of diagnostic panels for different leukemia and lymphoma diseases using conventional laboratory cytometers with longer wavelengths lasers as excitation sources.

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