Brilliant violet fluorochromes in simultaneous multicolor flow cytometry–fluorescence *in situ* hybridization measurement of monocyte subsets and telomere length in heart failure

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Conventional analytical methods to determine telomere length (TL) have been replaced by more precise and reproducible procedures, such as fluorescence *in situ* hybridization coupled with flow cytometry (flow–FISH). However, simultaneous measurement of TL and cell phenotype remains difficult. Relatively expensive and time-consuming cell-sorting purification is needed to counteract the loss, due to stringent FISH conditions, of prehybridization fluorescence by the organic fluorochromes conventionally used in the phenotyping step. Here, we sought to assess whether the newly developed Brilliant Violet (BV) dyes are valuable to specifically and simultaneously assess the distribution and telomere attrition of monocyte subsets circulating in the blood of a cohort of patients with heart failure. We performed flow–FISH on blood samples from 28 patients with heart failure. To differentiate among monocyte subsets, we used BV and conventional fluorochromes conjugated to antibodies against CD86, CD14, CD16, and CD15. We simultaneously assessed the TLs of the monocyte subsets with a telomere-specific peptide nucleic acid probe labeled with fluorescein isothiocyanate. The BV dyes completely tolerated the harsh conditions required for adequate DNA denaturation and simultaneously provided accurate identification of monocyte subpopulations and respective TLs. The presented protocol may be faster and less expensive than those used currently for purposes such as establishing associations among patient categories, disease progression, monocyte heterogeneity, and aging in the context of heart failure.

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Determination of telomere length (TL) is clinically valuable, since it reflects both the number of times the cell has divided and telomere attrition; both of these attributes are associated with the genetic instability, pathogenesis, and progression of a variety of human disorders. Current laboratory techniques for TL measurement include Southern blot, PCR, single TL analysis, and fluorescence *in situ* hybridization coupled with flow cytometry (flow–FISH). However, these methodologies have critical limitations; for example, they require large sample amounts and antibodies that are preferably conjugated to heat-stable fluorochromes, their analytical sensitivity is suboptimal when very short telomeres are assessed, and measurements among different samples vary excessively.¹

Flow–FISH is a methodology that has been thoroughly validated for clinical diagnosis, and it includes the option to analyze expression of specific surface antigens in either single cells or cell subsets.^{2,3} However, previous studies have revealed that combining cell phenotyping by flow cytometry with TL by FISH is difficult due to the harsh conditions required for hybridization of the telomere probe (ie, intense fixation and incubation at 82 °C). Relatively expensive and time-consuming cell subset purification (eg, by cell-sorting) is required to counteract the loss of fluorescence of the conventional, non-heat-stable, organic fluorochromes that are usually used in the immunophenotyping step. As an alternative, a small group of fluorochromes, including

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Quantum Dots (QD), cyanines, and Alexa Fluor dyes, have been reported to partially resist these stringent experimental conditions.^{4,5} However, it is difficult to conjugate QD with antibodies and therefore their use in detecting cell surface antigens is limited. To that end, researchers must usually isolate the cell subset of interest prior to performing flow-FISH.

The Brilliant Violet (BV) family of fluorochromes has unique optical properties to produce much brighter fluorescence signal than most of the traditional fluorescent dyes used in flow cytometry. Based on chemistry awarded with Nobel Prize, they are generated by about 1000 fluorescent monomeric subunits per macromolecule that act cooperatively along the entire length of the polymer backbone, resulting in emission of light with high molecular extinction coefficients and high quantum efficiency.^{6,7} Other advantages of this new class of dyes include synthetic modifications of their backbone structure to span the full range of the visible spectrum, and spectral compatibility with each other for multicolor simultaneous labeling.⁸

Heart failure remains a growing medical problem related to major hospitalization, mortality and poor prognosis worldwide.⁹ Although advances in diagnosis and treatment over last decades, many of the pathophysiological mechanisms and prognostic markers remain poorly investigated. In this context, the assessment of monocyte heterogeneity and/or ageing as potentially valuable predicting factors of categories and progression of heart failure is challenging. To that end, standardized instrument settings, reagents, and sample preparation and analytical protocols are required.

Thus, in the present study, we used the newly developed BV fluorochromes to specifically and simultaneously measure the distribution of circulating monocyte subsets and their respective TLs (RTLs) in a cohort of patients with heart failure.

MATERIALS AND METHODS

Study Population

We enrolled 28 consecutive patients diagnosed with heart failure and collected 3 ml of blood into EDTA tubes by conventional forearm venipuncture. The main clinical and demographic characteristics of the patients are summarized in Table 1. The study protocol was approved by the Clinical Research Ethics Committee of our institution, and it conformed to the principles outlined in the Declaration of Helsinki. Written informed consent was previously obtained from each subject. Samples were stored at room temperature and processed within 4 h after collection.

Multicolor Flow-FISH Procedure

Immunophenotyping

Whole-blood samples were lysed by incubating them with PharmLyse solution (BD Bioscience, San Diego, CA, USA) for 10 min. They were then washed twice with phosphate-buffered saline (PBS; Gibco Life Technologies/Invitrogen,

Table 1 Demographic and clinical characteristics of patients with heart failure

	N = 28
Age	64.9 ± 12
Male	20 (71.4%)
<i>Etiology</i>	
Ischemic	12 (42.9%)
IDCM	6 (21.4%)
Hypertensive	1 (3.6%)
Enolic	1 (3.6%)
Toxic	1 (3.6%)
Valvular	4 (14.3%)
Hypertrophic	2 (7.2%)
Others	1 (3.6%)
Duration heart failure (months)	28 (11–72)
LVEF	40 ± 12
<i>NYHA classification</i>	
I–II	22 (78.6%)
III–IV	6 (21.4%)
Diabetes mellitus	9 (32.1%)
Hypertension	14 (50%)
Previous myocard infarction	9 (32.1%)
<i>Treatment</i>	
ACEI/ARBII	25 (89.3%)
Beta-blockers	26 (92.9%)
MRA	17 (60.7%)
Loop diuretics	15 (53.6%)
Digoxin	8 (28.6%)
Ivabradine	8 (28.6%)
CRT	3 (10.7%)
ICD	4 (14.3%)

Abbreviations: ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter-defibrillator; IDCM, idiopathic dilated cardiomyopathy; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonists; NYHA, New York Heart Association.

Carlsbad, CA, USA) containing 0.5% bovine serum albumin (Miltenyi Biotech, Bergisch Gladbach, Germany). Cell concentration was determined by flow cytometry using Perfect-Count beads (Cytognos, Salamanca, Spain). We stained 1×10^6 cells with titrated amounts of antibodies conjugated to BV or Alexa Fluor dyes and specific to human CD86-BV605, CD14-BV785 (Biolegend, San Diego, CA, USA), CD16-BV421 and CD15-AlexaFluor647 (BD Biosciences) in 50 μ l of Brilliant Stain Buffer (BD Biosciences) for 15 min at room temperature (RT). We also comparatively tested a battery of commercially available antibodies against human

Table 2 BD Fortessa SORP optical configuration

Splitter	BP filter	Δ AutoFL
<i>Violet laser (405 nm)</i>		
750 LP	780/60	Low
670 LP	710/50	Medium
630 LP	660/20	High
595LP	605/40	High
550 LP	585/42	High
535 LP	560/40	High
505 LP	515/20	High
None	450/50	High
<i>Blue laser (488 nm)</i>		
750 LP	780/60	Medium
670 LP	705/70	Medium
635 LP	670/14	High
600 LP	616/23	High
None	575/26	High
<i>Green laser (532 nm)</i>		
750 LP	780/60	Medium
670 LP	705/70	Medium
635 LP	670/14	High
600 LP	616/23	High
None	575/26	High
<i>Red laser (640 nm)</i>		
735 LP	780/60	Low
690 LP	710/50	Low
None	660/20	Low

All fluorescence channels are named with the first letter of the laser (B, V, G, and R) and the central wavelength of the band-pass filter. Mirrors-splitters and band-pass filters are shown. Last column lists the level of increase in cellular autofluorescence in FISH protocol: High (> 5-fold), Medium (2- to 5-fold) and Low (< 2-fold) regarding background (non-FISH protocol) level.

CD8 coupled to BV421, BV510, BV570, BV605, BV650, BV711, or BV786 (Biolegend; BD Biosciences) or PE, PECy7, APC, PerCP, or BB515 (BD Biosciences) bound to compensation capture beads (OneComp eBeads; eBioscience, San Diego, CA, USA). After a brief centrifugation and two washes in PBS, cells were fixed with 6 mM bis sulfosuccinimidyl suberate (Sigma-Aldrich) for 30 min at 2–8 °C. The reaction was then immediately quenched by incubating with 1 M Tris buffer (pH 8.0) for 15 min at RT, and residual red blood cells were removed using FACS lysing solution (BD Biosciences) for 7 min at RT.

In situ hybridization

FISH was performed with the Telomere PNA kit (Dako, Glostrup, Denmark) in accordance with the manufacturer's instructions and using the lymphoblastic leukemia 1301 cell line as an internal control. An equal volume (300 μ l) of hybridization solution with or without FITC-labeled PNA probe (telomere-specific peptide nucleic acid probe) was added to sample and control tubes, respectively. Tubes were then hybridized for 10 min at 82 °C in a dry block heater (Grant Instruments, Cambridge, UK), and thereafter incubated overnight at RT. Tubes were washed twice in wash solution with previous incubation at 40 °C, and resuspended in 500 μ l of DNA-staining solution for 2–3 h at 2–8 °C. Samples were acquired by flow cytometry, and a minimum of 10 000 monocytes were collected in each sample tube. Correction for DNA ploidy of the blood sample *versus* the internal control was performed as described previously.¹⁰

Flow cytometry analysis

All samples were acquired on a Fortessa SORP flow cytometer (BD Biosciences) using 20 parameters (two scattering and 18 fluorescence detectors). The cytometer was equipped with four lasers with the following configurations: 100-mW 488 nm, 150 mW 532 nm, 50 mW 405 nm, and 100 mW 640 nm. All fluorescence channels were referred to by the first letter of the laser (B, V, G, and R), and the central wavelength of the band-pass filter (summarized in Table 2). We performed routine daily quality control tests with Cytometer Setup & Tracking Beads (BD Biosciences) in accordance with the manufacturer's instructions. The initial optimal voltage ranges and linearity for photomultipliers were selected using 6-peak Rainbow Calibration Particles and Unstained Comp-Beads (BD Biosciences), as described previously.¹¹ Owing to the increased cellular autofluorescence associated with FISH, PMT voltages were readjusted to obtain maximum sensitivity and resolution. A compensation matrix was applied by single-fluorochrome controls processed with the flow-FISH protocol. Data were analyzed with FACSDiva (BD Biosciences) and FlowJo (Treestar, Ashland, OR, USA) software.

We initially gated for G0/G1 cells of both leukocyte subsets and 1301 cells based on DNA content and then by scatter properties. Neutrophils and monocytes had similar scatter patterns after FISH; thus, monocytes were sequentially selected based on a CD86 *versus* CD16 plot, and a CD15 *versus* CD16 plot to gate out neutrophils. The gated monocytes were then analyzed for CD14 and CD16 expression. Clumped cells were excluded by propidium iodide (PI) area *versus* PI width plot. Each subset of monocytes and internal control cells was subsequently displayed on a plot comprising the FITC-labeled PNA probe on PI (B695-A) *versus* the PNA probe (B515-A), and the median fluorescence intensity (MFI) of the PNA probe was calculated. We determined the gate boundaries for CD14- and CD16-positive subsets with the appropriate fluorescence minus one controls. For bead-based comparative purposes, beads

were gated in forward and side scatter plots, and the MFIs of both negative and positive fluorochrome capture beads were analyzed.

The RTL value for each monocyte subset was calculated as the ratio between the MFI of each subset and the MFI of the control cells. Corrections were made for the DNA index of G0/G1 cells, as described previously.¹²

RESULTS

In these experiments, we assessed whether the newly developed BV fluorochromes were useful for flow-FISH. To that end, we attempted to simultaneously determine the identification of the three mononuclear cell subsets referred to as classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺) and nonclassical (CD14^{low}CD16⁺), as well as their respective RTLs in a cohort of patients with heart failure.

We first tested the thermal stability of BV dyes at 82 °C and the effects of the extensive fixation step that takes place in standard FISH protocols. We did so by analyzing the fluorescence emissions of different commercially available BV fluorochromes coupled to antibody capture beads. This allowed us not only to exclude the increased cellular autofluorescence that occurs during FISH but also to avoid changes in the antigen-antibody complex that can be provoked by the harsh conditions associated with FISH. The polymer-based BV dyes were heat stable and withstood the stringent conditions of hybridization with no degradation or loss of emission (Figures 1a–g). Additionally, the recently developed polymer-derived fluorochrome Brilliant Blue 515 showed the same thermal resistance (Figure 1h). By contrast, conventional organic or protein-based fluorochromes such as phycoerythrin, allophycocyanin, and peridinin-chlorophyll protein and related tandem fluorochromes were commonly completely degraded and their emissions abrogated during FISH (Figures 1i and k).

To determine if the stability of the BVs was sustained in a cell-based protocol, we incubated whole-blood leukocytes extracted from patients with titrated amounts of anti-human CD8-BV421, CD14-BV786, CD15-Alexa647, CD16-BV421, and CD86-BV605, and assessed them by standard FISH procedures. Although several of the photomultipliers that we evaluated in the violet laser line showed an increase from medium to high levels of cellular autofluorescence after fixation and incubation at 82 °C (most pronounced between wavelengths 515 and 660 nm; Table 2), discrimination among CD8⁺, CD14⁺, CD15⁺, CD16⁺, and CD86⁺ cell subsets related to the nonhybridization protocol was preserved (Figure 2). Table 2 shows the increased cellular autofluorescence associated with the FISH protocol. Increases in cellular autofluorescence were classified as High (>5-fold), Medium (2- to 5-fold), or Low (<2-fold) with respect to background levels. All of the BV dyes retained >60% of their pre-FISH Stain Index (data not shown).

We next assessed whether using BV-conjugated antibodies would allow us to discriminate among the different monocyte

subsets circulating in patients' blood by measuring cell surface expression of CD14 and CD16. We found that the BV-conjugated antibodies successfully discriminated among the monocyte subsets (Figure 3). Moreover, percentages of monocyte subsets measured before (after stopping BS3 crosslinking with Tris buffer) and after hybridization were very similar when measured with CD15-AlexaFluor647, CD16-BV421, CD86-BV605, and CD14-BV785, all of which are spectrally compatible with FITC-labeled PNA and PI (Figure 3). The calculated RTLs corresponding to each monocyte subset were 9.68 ± 2.21 , 9.76 ± 2.26 , and 9.96 ± 2.20 for classical, intermediate, and nonclassical monocytes, respectively (Figure 4).

DISCUSSION

In the present study, we evaluated whether BVs, a novel family of polymer-based fluorescent dyes, are valuable as heat-stable alternatives to conventional organic or protein-based fluorochromes in flow-FISH. We used the BVs to simultaneously measure the different circulating monocyte subpopulations and their respective TLs in blood samples from a cohort of patients with heart failure.

In general, current flow-FISH measurement of TL requires targeted cell purification (eg, by cell sorting).¹³ As an alternative, some studies have attempted to identify cell subsets by immunophenotyping and then applying TL calculations to all cells in a heterogeneous sample such as venous blood.¹⁴ Other studies have used a variety of heat-stable fluorochromes.¹⁵ In protocols in which targeted cells must be isolated prior to flow-FISH measurement of TL, it is also difficult to obtain the necessary number of cells for TL. Other limitations include thermal instability of conventional fluorochromes and high levels of cellular autofluorescence during the analytical process.¹⁶ Although other fluorochromes such as QD, cyanines, and Alexa Fluor are relatively thermally stable under the harsh conditions required for hybridization, their implementation is limited by the scarcity of appropriate reagents, the difficulty associated with conjugating them to antibodies, their excitation by several wavelengths of lasers,¹⁷ and the overlap of cellular autofluorescence and dye emissions.

Their polymeric structure and their benefit in multicolor staining panels suggest that the BV family of dyes may be successful alternatives to the brightest fluorochromes commonly used for immunophenotyping purposes, such as phycoerythrin and allophycocyanin. Moreover, their use enables the violet laser for polychromatic excitations. Additional major advantages of BV-based flow cytometry include their high solubility; lack of nonspecific binding; compatibility with intracellular staining; and efficient, stable conjugation to antibodies.⁶ BV dyes are more thermally stable than most low molecular weight fluorochromes, which lose their fluorescence under the intense fixation and high temperature (82 °C) required for DNA denaturation and hybridization of the fluorescent telomere probe.

Figure 1 Direct effect of FISH protocol on Brilliant Violet fluorochromes. Detection of BV421 (a), BV510 (b), BV570 (c), BV605 (d), BV650 (e), BV711 (f), BV786 (g) and BB515 (h) fluorescence signal using dye-labeled (right peak) and unlabeled (left peak) beads. Each BV signal is compared in FISH (red line histograms) and in non-FISH conditions (gray filled histograms). In contrast, conventional organic fluorochromes such as phycoerythrin (i), allophycocyanin (j) and peridinin chlorophyll (k) suffer complete degradation and subsequent emission abrogation under FISH conditions.

Based on the qualities described above, we hypothesized that BVs, as well as the new polymer-based dye Brilliant Blue BB515, would be appropriate alternatives to conventional fluorochromes in assays designed to simultaneously identify specific cell subpopulations (immunophenotyping) and hybridize probes to telomeres *in situ*. We also tested stabilization of the fluorochrome-antibody complex with the amine-to-amine cross-linker bis sulfosuccinimidyl suberate. This cross-linker is homobifunctional, water-soluble, noncleavable, and membrane impermeable; all of these properties are essential for BV-derived prehybridization fluorescence emission.¹⁸

To evaluate which BV dyes perform best in the detection of CD14, CD16, and CD86 expression in monocyte subsets and

TL calculation, we measured the levels of cellular autofluorescence induced by treatment and the spectral overlap of DNA dyes required for the identification of cell cycle phases. In our hands, PI staining decreases sensitivity in V560–V660 channels (data not shown), and this was taken into account. Moreover, fluorochrome selection must be optimal relative to expected antigen densities and dye efficiencies.¹⁹ Thus, in the present study, CD16-BV421, CD86-BV605, and CD14-BV786 were chosen for scrutiny.

Monocyte subsets play an important role in inflammation and innate and adapted immunity, and their contribution to cardiovascular diseases is currently being researched.^{20–23} In this context, our aim was to identify alternative fluorochromes for use in multicolor flow-FISH to differentiate

Figure 2 Comparative nonhybridization (gray filled histograms) and hybridization (red line histograms) staining protocols. Peripheral blood mononuclear cells (a) and monocytes (b) stained with CD14-BV786. Although increased cellular autofluorescence is shown using the FISH protocol (a), CD14⁺ cell staining is unaffected (a, b). Monocytes (c) and lymphocytes (d) stained with CD16-BV421. Both cell populations can be clearly discriminated from background. Peripheral blood mononuclear cells stained with CD86-BV605 (e). FISH protocol in a whole-blood sample using heat-stable BV421 and Alexa647-conjugated antibodies for specific detection of CD8⁺ lymphocytes and CD15⁺ neutrophils (f).

Figure 3 Representative analysis of whole-blood sample from a patient with heart failure. Monocyte subsets showing different expression levels of CD14 and CD16 cell surface antigens under prehybridization (left contour plot) and post-hybridization (right contour plot) conditions. Both plots depict a similar fluorescence intensity and frequency pattern of the different monocyte subsets.

Figure 4 Gating strategy for the RTL analysis in monocyte subsets. G0/G1 cells were selected in DNA content histogram (a) and then gated by scatter properties (b, monocytes and f, 1301 cell line). CD86 versus CD16 (c) and CD15 versus CD16 (d) plots were used to specifically select monocytes; thereafter both CD14 and CD16 expression were analyzed (e). (h) Hybridization of FITC-labeled PNA probe and control (without probe) in monocyte subsets based on the CD4 and CD16 expression and in 1301 cell line, which was used as internal reference control (g).

among monocyte subpopulations and quantify their RTLs. Moreover, fixation steps can lead to loss of differentiation based on scatter properties, and monocyte subsets expressing CD14 and CD16 antigens are not easily separated from other CD16⁺ leukocytes such as neutrophils. To address this concern, we confirmed that discrimination between monocytes and neutrophils requires the employment of additional cell surface antigens, such as an exclusion marker for neutrophils (eg, CD15).

Taken together, our results show that BVs used in flow-FISH can facilitate accurate, specific, relatively inexpensive (approximately 3-fold less expensive), and faster measurement of circulating monocyte subsets and their RTLs in the context of heart failure. Therefore, our technical protocol may be used to further explore important associations among patient categories, disease progression, monocyte heterogeneity, and aging in series that comprise broader ranges of patients than those assessed in conventional studies.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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