Feasibility of Measuring Autofluorescence of Red Blood Cells Utilizing a Novel Flow Cytometer to Define Iron Deficiency Patients

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Several different indices have been proposed for differentiating microcytic anemia. These indices are based on RBC parameters which are provided from a hematology analyzer. Zinc protoporphyrin IX (ZnPP) is also used for the screening of microcytic anemia, especially for iron deficiency anemia (IDA), because ZnPP and metal-free protoporphyrin IX (PPIX) are found at elevated levels in RBCs if heme production is inhibited by lack of iron. It is well known that ZnPP and PPIX in red blood cells emit autofluorescence through excitation by blue-violet light. The aim of this study is to assess new rapid and precise method for screening IDA with autofluorescence from red blood cells detected by customized XN hematology instruments.

The customized XN used in this study has two kinds of laser. In addition to standard 640nm laser, the instrument was equipped with a 405nm laser. Further, two additional detectors, one for detecting 405nm forward light scatter signals and another for detecting the fluorescence excited by 405nm laser, were mounted on the instrument. When RBC is excited by 405nm laser, autofluorescence from RBC in IDA samples can be detected on the customized XN.

The intensity of autofluorescence from red blood cells was measured on the customized XN for a set of 16 non-anemia samples and 19 samples with IDA. Autofluorescence signal intensity in IDA samples was much higher than in non-anemia samples. Further, we found a positive correlation between autofluorescence and ZnPP levels.

The customized XN can provide information of iron deficiency as well as common hematology parameters. We expect that this inexpensive and simple approach will be a cost effective alternative to current procedures particularly in developing countries.

Key Words Autofluorescence, Iron Deficiency Anemia, Thalassemia, Zinc Protoporphyrin, New Instrument

INTRODUCTION

Anemia is a very common disease entity seen at all ages and in all ethnicities. Multiple causes may underlie or lead to anemia. The most common and most practical approach for anemia work-up is its classification by measuring erythrocyte (red blood cells) size and average hemoglobin content. Thus, anemias are differentiated into microcytic, normocytic or macrocytic appearances. In the case of microcytic anemia, the most prevalent causes are iron deficiency anemia (IDA) and either alpha- or beta-thalassemia (THA) and the combination thereof.

Several different indices (Mentzer [1)], England and Fraser [2)], and Green and King [3)], etc.) have been proposed for differentiating microcytic anemia. These indices are based on RBC (RBCs, red blood cells; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; etc.) which are provided from a hematology analyzer. In addition, different work-up algorithms have been proposed and are in clinical use around the world. In some of those algorithms, zinc protoporphyrin IX (ZnPP) is also used for the screening of microcytic anemia [4)]. If heme production is inhibited by lack of iron, ZnPP and metal-free protoporphyrin IX (PPIX) are found at elevated levels in red blood cells.

Due to diagnosis of iron deficiency with different treatment requirements, a rapid and precise, furthermore inexpensive and simple diagnosis is most valuable for routine laboratory testing, especially for developing countries. The purpose of this study was to test a newly developed instrument for reliable, rapid and cost efficient anemia diagnostics. We performed a pilot study to test feasibility of the novel approach in a small but very well defined patient collective.

MATERIAL AND METHODS

Blood samples

During a period of one month, 35 residual K_2EDTA anticoagulated samples were evaluated. Samples included 16 non-anemia samples and 19 samples with iron deficiency anemia. Non-anemia samples, in which blood cell counts and biochemical iron test were within the reference range, were obtained in the course of routine analysis. RBC indices, iron, ferritin and ZnPP in ***Table 1*** were measured by XN-9000 (Sysmex, Japan), Dimension Vista 1500 (Siemens Healtneers, Germany) and hematofluorometer (Aviv Biomedical, Lakewood, NJ, USA) respectively. Samples with iron deficiency were further divided into the known three stages according the iron metabolism parameters (iron, ferritin, ZnPP etc.) based on routine workflow. The classification comprised Stage 1 of IDA: only ferritin is out of normal range; Stage 2 of IDA: iron, ferritin and ZnPP are out of normal range; Stage 3 of IDA: all parameters including RBC indices are out of normal range. Normal ranges for each parameter are summarized in ***Table 1***.

Table 1 *Hematological and biochemical parameters*
As a result of the routine workflow, iron, ferritin, ZnPP in some samples were not measured.

			Non-Anemia	IDA		
				Stage1	Stage2	Stage3
parameter	Normal range*	Total sample number	16	5	2	12
RBC, 10^{12}/L	3.9-5.9	Measurement sample number	16	5	2	12
		mean	**4.61**	**4.33**	**4.09**	**4.34**
		sd	0.36	0.85	0.06	0.78
Hgb, g/dL	12-17	Measurement sample number	16	5	2	12
		mean	**13.9**	**12.6**	**11.1**	**10.0**
		sd	0.9	2.6	0.3	1.9
MCV, fL	80-90	Measurement sample number	16	5	2	12
		mean	**91.4**	**88.9**	**87.0**	**77.0**
		sd	4.2	3.7	1.3	10.4
MCH, pg	27-33	Measurement sample number	16	5	2	12
		mean	**30.3**	**29.1**	**27.2**	**23.2**
		sd	1.6	1.3	0.3	3.5
MCHC, g/L	31-36	Measurement sample number	16	5	2	12
		mean	**33.1**	**32.7**	**31.2**	**30.1**
		sd	0.8	1.1	0.1	1.6
Iron, μmol/L	< 5	Measurement sample number	13	5	2	12
		mean	**15.1**	**14.4**	**5.3**	**4.4**
		sd	8.3	5.5	1.3	1.8
Ferritin, μg/L	< 30	Measurement sample number	16	5	2	11
		mean	**105.3**	**13.7**	**11.9**	**8.6**
		sd	79.3	2.6	3.3	5.8
ZnPP, μg/dL	< 50	Measurement sample number	7	4	2	8
		mean	**33.9**	**51.2**	**147.0**	**154.8**
		sd	41.0	31.0	15.6	125.5

Instrument and optical system

We used a Sysmex XN hematology instrument customized for this study. The XN Module analyzer was introduced by Sysmex in 2011 as a flagship model of the hematology analyzer series. The customized XN has two kinds of laser (XN with Dual Wavelength Laser: XN-DWL). The optical system on standard XN instruments only employs a red diode laser producing a light beam of 640nm. The XN-DWL is equipped with an additional blue-violet laser which can produce a laser beam at 405nm.

The XN-DWL has five signal detectors. Three detectors for 640nm red forward scattered light (R-FSC), 640nm red side scattered light (R-SSC) and side fluorescence excited by 640nm red laser (Rex-SFL) have same specifications as standard XN instruments. Additionally, new detectors for 405nm blue-violet forward light scatter (V-FSC) and fluorescence excited by 405nm blue-violet laser (Vex-SFL) are attached to complete the customization process.

The specifications of 405nm laser and the detector for Vex-SFL are designed for detecting autofluorescence from red blood cells. The wavelength of 405nm laser is close to the absorption wavelength of PPIX, reported as approximately 400 nm [5]. The power of blue-violet laser is 60 mW to detect autofluorescence from red blood cells sufficiently. The optical filter for Vex-SFL can transmit wavelengths longer than 654 nm. This specification of the filter is to block the light from the 640 nm laser and transmit the light from PPIX which emits autofluorescence at wavelengths longer than 654 nm [6]. This new optical configuration enables the detection of autofluorescence at wavelengths longer than 654nm from red blood cells excited by 405nm laser.

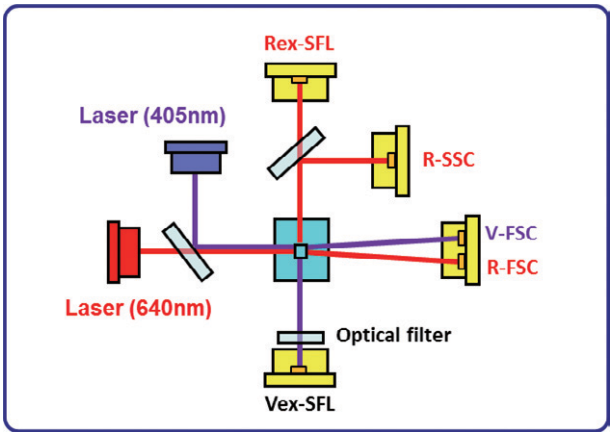


Fig. 1 *Optical configuration of XN-DWL (XN with Dual Wavelength Laser)*
The instrument is equipped with two laser sources and five detectors. 405nm laser, detector for V-FSC (405nm violet forward light scatter) and detector for Vex-SFL (fluorescence excited by 405nm blue-violet laser) are additional from standard XN instrument. Optical filter in front of Vex-SFL detector is optimized for detecting autofluorescence from RBC.

Reagents

Red blood cells were diluted by XN reagent, CELLPACK DCL, without dye for reticulocyte. The measurement mode of the reticulocyte (RET) channel on the XN instrument was utilized for detecting autofluorescence from red blood cells. In the RET channel on the XN instrument, blood cells were stained by a polymethine dye that binds to cytoplasmic RNA to permit reticulocyte counting. However, this staining protocol was modified for the autofluorescence detection.

Erythrocyte analysis

Erythrocyte scatterplot (***Fig. 2***) shows the position of RBC and platelets (PLT). Vex-SFL is plotted along the x-axis and V-FSC is plotted along the y-axis. Forward scattering intensity is correlated with cell size in general, therefore upper population on the scatter plot shows red blood cells and lower population shows PLT in ***Fig. 2***. We created the specific gating (AF gate) on the scatterplot for detecting the presence of red blood cells emitting autofluorescence. Lower limit (LT) of the AF gate along x-axis was determined by the population of red blood cells without autofluorescence in healthy samples. We measured ten healthy samples in advance and calculated average and standard deviation (SD) of red blood cell population along the x-axis. For the calculation, the value of average plus three SD was defined as the lower limit of the AF gate. Y-axis range for the AF gate was determined so that most of red blood cells could be in this gate. Another specific gating for counting the number of red blood cells was also determined as the "RBC gate". Y-axis range for the RBC gate is same as for the AF gate. According to these gates, autofluorescence rate (AF%) was calculated using the following formula:

$$\text{Autofluorescence rate (AF\%)} = \frac{\text{Cell number in AF gate}}{\text{Cell number in RBC gate}} \times 100$$

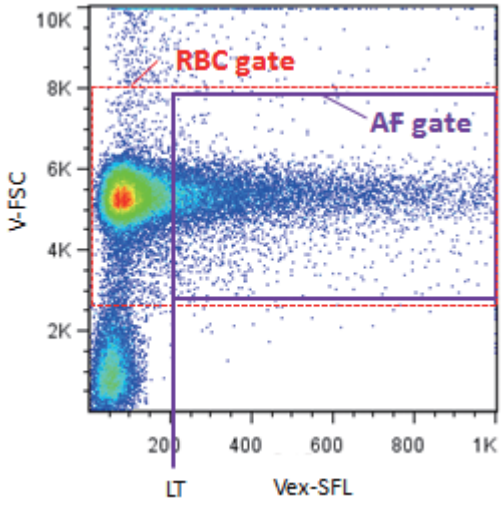


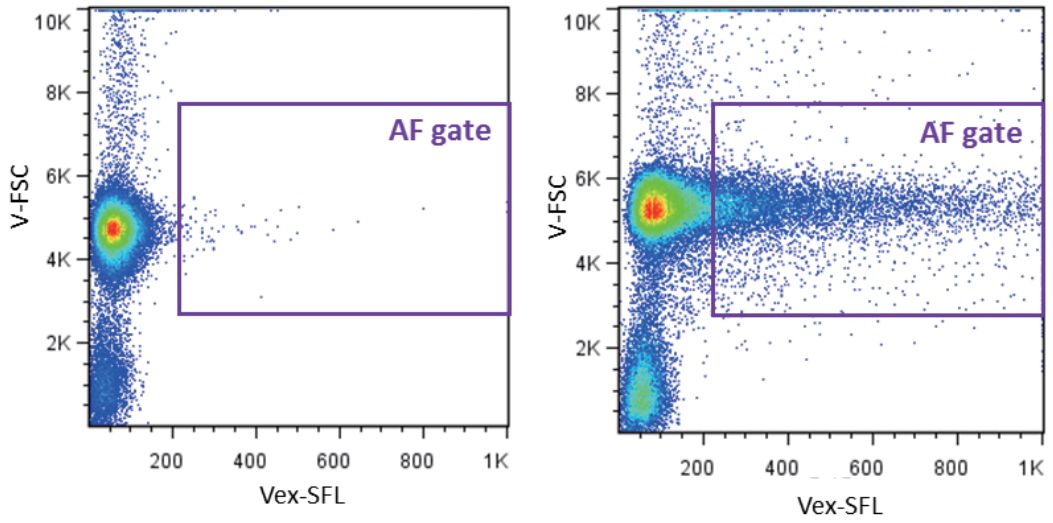
Fig. 2 *Definition of AF gate for calculating autofluorescence rate*

X axis is Vex-SFL (fluorescence excited by blue-violet laser) intensity; Y axis is V-FSC (405nm forward light scatter) intensity. Lower limit (LT) of the AF gate along x-axis is determined by the population of RBC without autofluorescence in healthy samples.

RESULTS

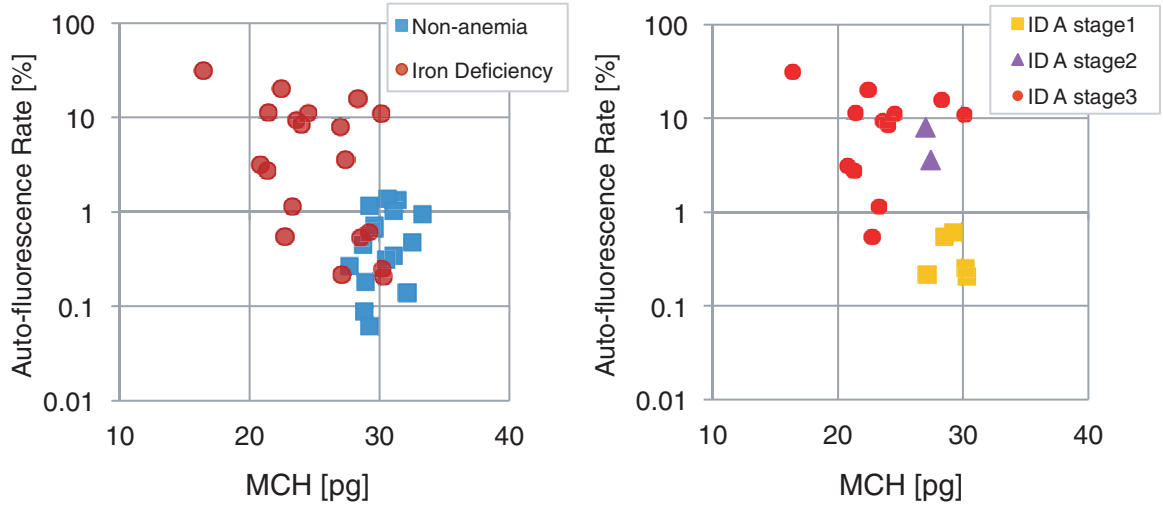
Scatter plots of two different samples are shown in ***Fig. 3***. We found that many cells are observed in the AF gate on the scatter plot of IDA sample. According to the signal intensity of V-FSC, it is suggested that these cells are red blood cells.

Looking at the AF% as a function of MCH in ***Fig. 4***, we found that AF% of samples with iron deficiency tends to be higher than AF% from non-anemia samples. In our results, five iron deficiency samples are overlapped with population of non-anemia samples on the scatter plot. However, all these five iron deficiency samples are stage 1 IDA samples. It is suggested that the intensity of autofluorescence from red blood cells is dependent on the severity of iron deficiency. MCH was selected as the most effective parameter among several RBC parameters in order to differentiate between non-anemia and IDA on the scatter plot.



(a) Non-anemia *(b) IDA*

Fig. 3 *Scatter plot differences between non-anemia sample and IDA sample*
The populations of RBC and PLT can be separated along the Y axis. (a) Non-anemia (AF%=0.24%), (b) Iron deficiency (AF%=18.02%). X axis is Vex-SFL (fluorescence excited by blue-violet laser) intensity; Y axis is V-FSC (405nm forward light scatter) intensity.



(a) Non-anemia and IDA *(b) IDA Stage1, 2 and 3*

Fig. 4 *Differentiation between non-anemia and iron deficiency anemia.*
X axis is MCH which is measured by routing hematology instrument in the laboratory; Y axis is AF% which is calculated from the results obtained from the XN-DWL. All results are plotted on Figure 4 (a), whereas only IDA samples further separated to 3 stages of IDA are plotted on Figure 4(b).

Finally, significance levels of AF% between non-anemia and IDA are calculated and presented in ***Fig. 5***. These significance levels were calculated by Mann-Whitney U test as a nonparametric statistical analysis. We found that IDA samples and non-anemia samples are differentiated sufficiently by AF%.

DISCUSSION

In this study, we found that the XN-DWL, which is a modified version of the Sysmex XN hematology instrument, could detect autofluorescence from red blood cells. Measured AF% in IDA is much higher than in non-anemia red blood cells. However, variation of AF% in IDA samples is larger than in non-anemia samples. It is suggested that this result is related to the extent of the iron deficiency. ***Fig. 6*** shows the correlation between ZnPP and AF% in IDA samples. ZnPP was measured by hematofluorometer, which is used for routine laboratory testing.

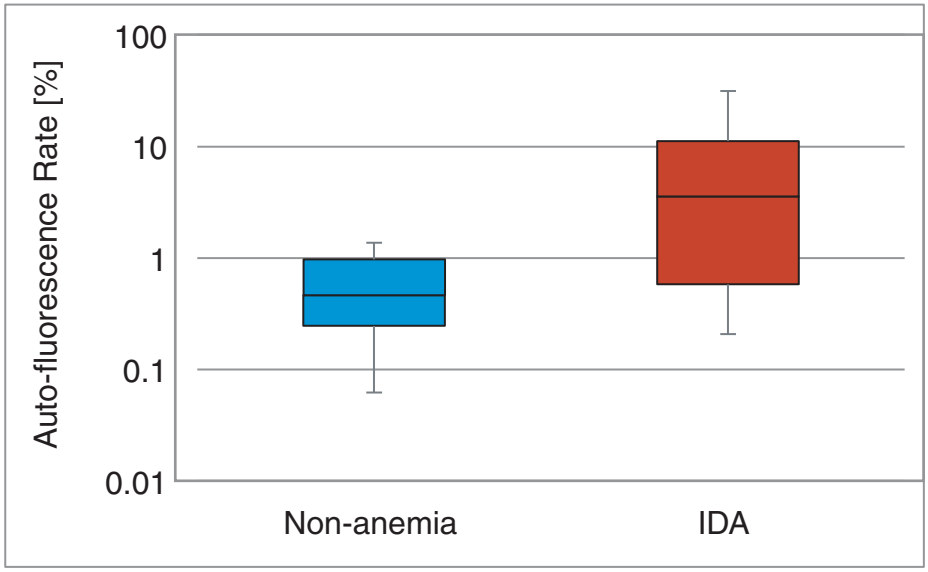


Fig. 5 *Significance levels between Non-anemia and IDA.*

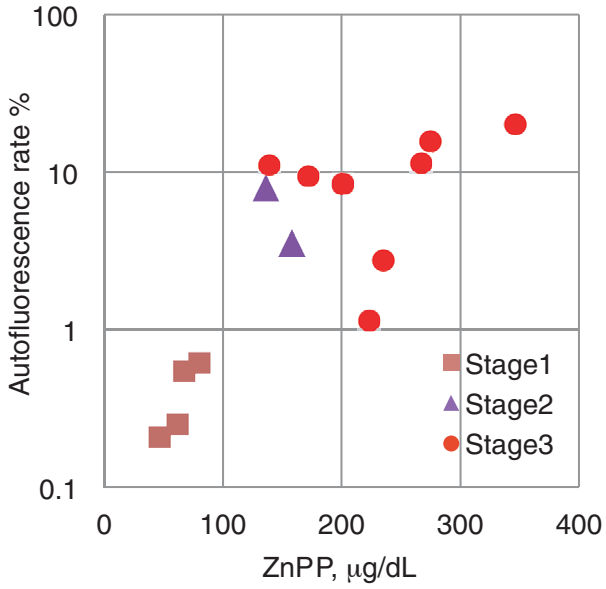


Fig. 6 *Correlation between ZnPP and autofluorescence in IDA*
AF% increases with increasing ZnPP in IDA patients and AF% increases as the stage of iron deficiency progresses.

We found that AF% increases with increasing ZnPP in IDA patients and AF% increases as the stage of iron deficiency progresses. It is known that ZnPP and free PPIX emit autofluorescence whereas heme (PPIX combined with iron) does not. The absorption maximum and autofluorescence of ZnPP are reached at 425 nm and 593 nm respectively [5, 7]. The absorption maximum and autofluorescence emission of free PPIX, however, occur at 397 nm and 627 nm respectively [5, 7-10]. According to the specification of the XN-DWL, autofluorescence from both proteins could be detected by this instrument. Based on these facts, AF% might be a new parameter for measuring the extent of iron deficiency, analogous to current analysis of ZnPP. AF% could be provided from a single hematology analyzer together with other well-known and commonly used hematological parameters. This new approach could be an effective and cost efficient tool for testing of microcytic anemia in developing countries as only one instrument and method is necessary.

This feasibility study demonstrates that the novel method is possibly capable of diagnosing iron deficient anemias. Further, it might be possible to differentiate the three known iron deficiency stages 1, 2 and 3 or at least 2 and 3 from normal, non-iron-deficient erythrocytes.

In addition, some preliminary results led us assume that thalassemia expresses lower amounts of ZnPP and PPIX. It is necessary to study whether our findings on iron deficiency determination and differentiation of IDA can be confirmed. Further, it would be of interest to study patients with THA using this novel technology. Thus, a follow-up study with large and well defined patient populations should be initiated.

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