Evaluation of Confocal Microscopy as a Diagnosis Tool on Red Blood Cell Diseases

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Abstract

In hereditary spherocytosis, mutations in red blood cell membrane proteins result in an overly rigid, misshapen cell whose deformability when traveling through the blood vessels is lost, causing severe anemia and splenomegaly, jaundice, and gallstones. In thalassemia, mutations in the globin genes can cause also severe anemia, skeletal and growth deficits and iron overload. Diagnosing these entities can be difficult due to the coexistence of other causes of anemia and blood transfusions, so complex molecular tests are required. In order to avoid these, we explored the possibility of using spectral confocal microscopy as a diagnostic tool for hereditary spherocytosis and thalassemia in pediatric patients. The red blood cell membrane was stained with different color dyes and immunolabels, to identify possible membrane defects expressed as differences in color and shape under a Leica TCS SP8. Staining the membrane and nuclei with lipophilic fluorescent dyes permitted the precise assessment of cell shape.

Keywords: *hereditary spherocytosis, confocal microscopy, spectral imaging, cell morphology, membrane stains*

INTRODUCTION

Diagnosing blood diseases during childhood is crucial since they can cause very severe syndromes, affecting the correct development of several organs. Some of such diseases are hereditary spherocytosis (HS) and thalassemia.

HS is caused by mutations in at least five genes that code red blood cells (RBCs) membrane proteins, according to Eber and Lux (2004). These proteins transport molecules into and out of cells, attach to other proteins, and in general, they maintain cell structure. The misshapen RBCs in HS, called spherocytes, have a characteristic spherical shape and they are too stiff to circulate through capillaries, so they are removed from circulation and taken to the spleen, where they undergo hemolysis. The shortage of RBCs in circulation and the abundance of cells in the spleen are responsible for the signs and symptoms of HS. There are four forms of HS, which are distinguished by the severity of signs and symptoms. They are known as the mild form (20-30% prevalence), the moderate form (60-70% prevalence), the moderate/severe form (10% prevalence), and the severe form (3-5% prevalence). People with the mild form may have very mild anemia or sometimes have no symptoms. Moderate HS usually appear in childhood and causes anemia, jaundice, splenomegaly, and gallstones. Individuals with the moderate/severe form have all the of the moderate form but the anemia becomes severe. The severe form causes life-threatening anemia that requires frequent blood transfusions to replenish their red blood cell supply, splenomegaly, jaundice, and a high risk for developing gallstones. Some

individuals with the severe form have short stature, delayed sexual development, and skeletal abnormalities, according to Mariani et al. (2008).

Usually, the diagnosis of HS is based upon identifying the abnormal RBCs by their round shape and darker color under a conventional optical microscope; the darker color is caused by a greater amount of hemoglobin on a smaller cell volume. Also upon indices of a blood test that include a complete blood cell count (CBC), reticulocyte count and mean corpuscular hemoglobin concentration (MCHC) which is usually elevated. Other kinds of common indicators are the lactate dehydrogenase (LDH) level and the fractionated bilirubin level. In order to rule in or out other diseases that may be a cause of abnormal RBCs, a bone marrow biopsy can be also carried out. Despite these resources, sometimes these entities are not easy to diagnose. For very much equivocal cases, more complex studies of membrane proteins or even genetic studies need to be undergone, as stated by de Oliveira and Saldanha (2010).

In thalassemia, mutations result in a quantitative reduction in the rate of synthesis of the globin chains which form hemoglobin (alpha or beta-globin chains), referencing Desousky et al. (2009). Clinical manifestations of thalassemia syndromes range from no symptoms in asymptomatic carriers to serious abnormalities that include severe anemia, extramedullary hematopoiesis, skeletal and growth deficits and iron overload. The severity of the clinical features correlates with the number of functioning globin genes that are lost, according to Provan et al. (2015). Thalassemia is diagnosed by means of traditional techniques such as conventional microscopy and complete blood count analysis and, similarly to HS, sometimes genetic studies are needed to diagnose carriers and moderate forms of the disease.

Currently, confocal spectral microscopy is an imaging modality that is widely used for spectral, color and morphological analysis of any biological sample. It captures the light on a sample volume from a specific plane of focus with nanometric precision and from a wide spectral range, then by collecting several axial cuts, tridimensional (3D) color maps of the functional information of the cell can be build. Therefore, this imaging modality allows the sequential study of the cell structure and molecular components, arising from reflectance, autofluorescence, or even fluorescence by staining different cellular components with the use of extrinsic fluorescent probes.

Some related works stain the plasma membrane uniformly with lipophilic plasma membrane dyes not specific for any protein, for the assessment of cellular shape, not stablishing any differences in color or spectral content. Khairy et al. (2008) analyzed the 3D shapes of RBCs using the fluorescent color dyes Calcein and Vybrant Dil to label the cytoplasm and plasma membrane, respectively. These RBCs where collected from a healthy subject and chemically induced to acquire the characteristic shapes from pathologies such as spherocytosis. By means of mathematical simulations, authors were able to relate each shape with the amount of shear energy in the membrane associated cytoskeleton. Rappaz et al. (2008) compared the morphological values of RBCs, such as the mean cell volume and the RBC distribution width, obtained from different imaging techniques including spectral confocal microscopy. To perform volume assessment of RBCs using this technique, cells were labeled with the Vybrant Dil dye and excited at 561 nm, collecting the emission from 580 nm to 700 nm. They concluded that among all methods, digital holographic microscopy and spectral confocal microscopy, where the ones that led to a more precise volume estimation. In this context, and following our previous study in patients with thalassemia, referenced in Rey-Barroso et al. (2020), we have conducted experimental assays to analyze healthy and diseased RBCs in HS patients by means of spectral confocal microscopy, for the future development of simpler and more effective protocols of diagnosis.

MATERIALS AND METHODS

Subjects

Blood samples from 9 pediatric patients were evaluated, 5 males and 4 females between 1 and 18 years old, including patients with different forms of HS and also 3 healthy children as controls. Also the samples of 2 progenitors with HS were collected. Two parallel assays were conducted: a first, more complex immunostaining protocol by which certain proteins in the cell membrane were specifically targeted by means of antibodies (Ab) with different fluorescent probes attached; and a second study preserving the living cell and adding lipophilic membrane stains as done in the state of the art. The first study sought to reveal spectral/color variations in the Ab fluorescence intensity along the membrane; the second sought to label the membrane uniformly on fresh samples to be able to preserve the morphology of RBCs for its analysis. RBC indices (Table 1) for each sample were obtained using an ADVIA 2120i hematology analyzer (Siemens Healthcare Diagnostics Inc., Erlangen, Germany) within 2 hours after blood collection. Pediatric patients with HS are labeled as $HS_{1\text{-}6}$, the progenitors that were also included in the study are labeled as HS_{P1} and HS_{P2}. Healthy children used as control subjects are labeled as $HS_{C1\text{-}3}$. Subjects HS_5 and HS_{P1} suffered from severe forms of HS and had to be splenectomized to ensure the disease best evolution and to improve their quality of life.

HS severity	Hb (g/dL)	MCV (fl)	MCHC (pg)	%RETI	%HPR	%MICRO
Severe	7.2	70.5	34.8	15.28	8.7	29.6
Moderate	11.4	88	34.9	9.03	9.2	2
Mild	14.5 ±1.50	82.8 ±8.35	36.0 ±0.81	3.3 ±1.49	9.5 ±7.59	3.9 ±5.11
Control	13.7 ±0.71	85.4 ±4.87	34.1 ±0.57	1.9 ±0.21	0.7 ±0.61	1.0 ±0.67

Table 1: Mean and standard deviation of severe (HS_1), moderate (HS_3), mild (HS_2, $HS_{4\text{-}6}$, $HS_{P1\text{-}2}$) and control ($HS_{C1\text{-}3}$) subjects for hemoglobin (Hb) in grams per deciliter (g/dL); medium corpuscular volume (MCV) in femtoliters (fL); medium corpuscular hemoglobin (MCHC) in picograms (pg) and percentage of reticulocytes (RETI), hyperchromic cells (HPR) and microcytic cells (MICRO).

HS is inherited in an autosomal dominant pattern for about 75% of cases, so one usual criteria for diagnosis of a child is that one of the progenitors is also affected by HS, according to Iolascon and Avvisati (2008). In general, the degree of severity of the disease is related with the degree of hemolysis, with parameters like hemoglobin in blood and reticulocytes count. A subject with severe HS is expected to have Hb (g/dL) < 8, reticulocyte count (%) > 10. In addition, the number of spherocytes present in the blood sample related with the hyperchromic cell count (%HPR) is an indicator of the severity of HS. Mutations in proteins responsible for HS occur predominantly in ankyrin, band 3, and beta-spectrin, and almost every family has a unique mutation. In this work, the genetic studies that were carried out on a few subjects did not reveal any differences over known mutation places.

Sample preparation

Whole blood of patients with lithium heparin as an anticoagulant agent to avoid sample corruption was used to conduct both assays; for the immunostaining protocol, band 3 was the target protein to label. Cells had to be fixated with paraformaldehyde 2%, and then blocked with PBS-BSA 1

%. Then, they were incubated with a primary monoclonal mouse Ab, BRIC 200 specific for biding the extracellular domain of band 3. The fluorochrome molecule attached to the Ab was iFluor488 (International Blood Group Reference Laboratory, Bristol, UK) with a fluorescent green emission at 525 nm. Finally, samples were mounted over slides with cover slips to acquire 3D stacks of the fluorescence distribution of the Ab over the membrane.

For the study of the cells in-vivo, blood samples were used in fresh without the addition of solvents or any other kind of saline solution. Samples were loaded into cell culture dishes CELLview™ (Greiner Bio One GmbH, Courtaboeuf, France), which incorporate a cell-adhesion layer which is critical to prevent cellular movement when capturing an image stack of living RBCs. The cell membrane and nuclei were stained respectively with fluorescent color dyes CellMask™ Deep Red with a fluorescent red emission at 666 nm, and Hoechst 33342, which is a fluorescent staining with blue emission wavelength at 461 nm (Thermo Fisher Scientific Inc., Waltham, MA USA). With temperature and CO_2 control inside the microscope cabin, the 3D stacks of the samples were acquired to evaluate the number of spherocytes in the blood samples of the aforementioned patients.

Spectral confocal microscopy evaluation

The samples were analyzed under a Leica TCS SP8 confocal microscope with stimulated emission depletion (STED) 3× super resolution (Leica Microsystems GmbH, Mannheim, Germany). It incorporates hybrid detectors capable of detecting signals with high sensitivity coming from RBCs from 400 nm to 790 nm. The microscope incorporates two lasers for excitation: a diode laser with an emission of 405 nm and a white laser that emits from 470 nm to 670 nm, combined with an acoustic-optic tunable filter (AOTF).

To collect the spectral emission of the stained RBCs, a 63× (NA 1.4, oil) plan-apochromatic objective was used and a 1 airy unit (AU) pinhole was set. For the case of the immunostained cells, BRIC 200 whose fluorescent probe was iFluor488 was excited with the white laser (488 nm) and detected in the 505–580 nm range. For the case of the in-vivo analysis, Hoechst 33342 DNA label was excited with a blue diode (405 nm) and detected in the 420–470 nm range. The CellMask™ dye was excited with a white laser (633 nm) and detected in the 645–775 nm range. An image acquisition sequence xyλz to acquire the 3D stacks of the fluorescence distribution of the color dyes was performed, using hybrid detection. Finally, images were deconvoluted using software Huygens Professional Software v17.10.0p8 (SVI, Leiden, The Netherlands) in order to improve image quality. Images of both assays were generated using the Leica LAS X software (Leica Microsystems, Wetzlar, Germany).

RESULTS AND DISCUSSION

For the first immunostaining assay, the fluorescent 3D image stacks with labeled band 3 were recovered. From 5 to 10 fields were acquired over the whole blood samples of each subject. The membranes were stained with a bright green color and the projection sum of the images of each stack was calculated to retrieve the mean fluorescence of the samples. In Figure 1, we represented some of the acquired fields for four of the subjects, but all of them led to very similar values of mean fluorescence. This means that there are no spectral/color variations for this particular protein among different HS forms or healthy patients in the 505–580 nm range. Nevertheless, a more complex immunostaining protocol could be stablished to enhance the cellular structures and get a greater detail over the membrane for the analysis of the Ab distribution. On the contrary, in the experimental assays conducted on thalassemia, in which we studied the autofluorescence of the samples, living RBCs were

excited at 405 nm and their emission was collected in the spectral range from 425 nm to 790 nm. Differences at 628 nm and 649 nm emission peaks, mainly, were found due to the phorphyrin presence in blood due to heme group degradation, Rey-Barroso et al. (2020).

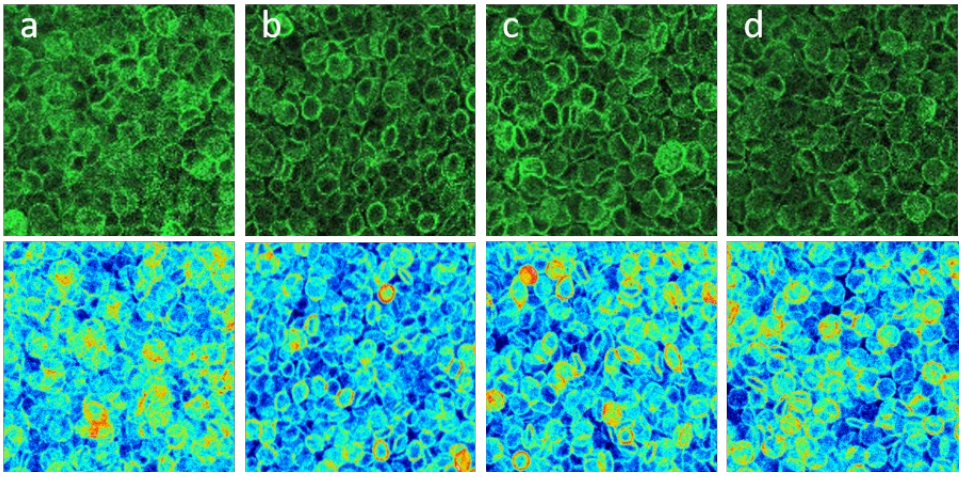

Figure 1: A confocal spectral image from the 3D stack acquired at 505-580 nm of images for a field (top) and its projection sum image (bottom) of subjects (a) HS_{C2}, image of field 03 and projection sum, (b) HS_2, image of field 04 and projection sum (c) HS_6, image of field 02 and projection sum, (d) HS_{P2}, image of field 03 and projection sum.

For the in-vivo assay, a set of high-quality 3D image stacks were acquired for 5 to 10 fields over the whole blood samples of each subject. The membranes were stained with a bright red color that permitted the precise assessment of cellular shapes when evaluating the fluorescence of the CellMask™ dye in the 645–775 nm range (Figure 2). Among them, healthy RBCs presented the characteristic biconcave shape and a central pallor region. Spherocytes, smaller and rounder RBCs, were also present in the samples from diseased individuals. They are hyperchromic RBCs since they contain more hemoglobin than those normal in relation to cell volume, which is < 80 μm^3. Neutrophils were distinguished from RBCs for its bright blue stained nuclei as a consequence of the Hoechst 33342 fluorescent color dye. From the image stacks in Figure 2, we can identify all the aforementioned cellular types and relate data in Table 1 regarding the percentage of hyperchromic cells with the number of spherocytes population for each subject. That is why a higher hyperchromic cell count is related to HS. Unlike conventional optical microscopy, confocal spectral microscopy allows the precise and unequivocal 3D assessment of cell shape.

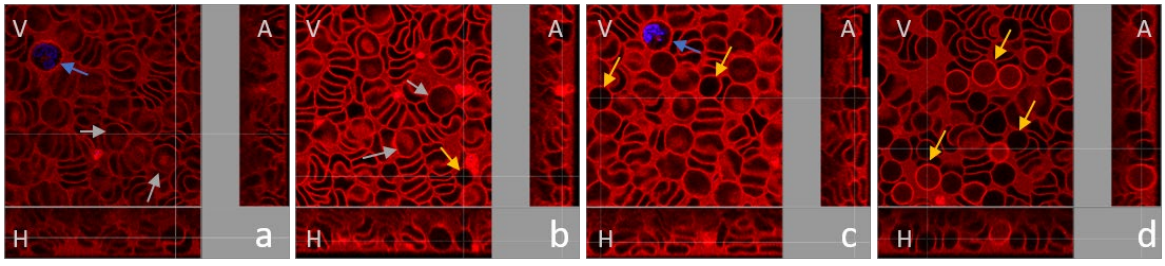

Figure 2: Confocal spectral image stacks of one field on which a vertical (V), a horizontal (H) and an axial (A) section has been selected in (a) HS_{C2}, where no spherocytes are visible and %HPR = 0.4, in (b) HS_4, where just some few spherocytes (yellow arrows) are visible and %HPR = 5.3, in (c) HS_6 where some more spherocytes are visible and %HPR = 9.2, in (d) HS_{P2}, where there is a high number of spherocytes present and %HPR = 22.9. Neutrophils are those cells with blue nuclei (blue arrows). Normal RBCs are labeled with gray arrows.

CONCLUSIONS

A 3D image-based method for the evaluation of HS has been presented. Spectral confocal imaging has been shown to be a direct way to precisely identify the number of spherocytes on patient's samples, instead of making an estimate with the hyperchromic cell count. In this work, we have proven that simply color labeling the membrane and nuclei of blood cell types, patients' samples with some degree of HS are clearly different from healthy patients' samples when evaluating the morphology of RBCs.

Research efforts are now focused on trying to label the more specific proteins in the membrane of RBCs that are known to be affected in HS and find spectral signatures to better discriminate between healthy RBCs and those suffering from HS. For this purpose, protocols on color labeling and preservation of this kind of samples, which are corrupted in a very short time window, are being investigated.

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