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Original Research Paper

Validating the measurement of red blood cell diameter in fresh capillary blood by darkfield microscopy: A pilot study

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ABSTRACT

Objectives: Analysis of fresh capillary blood using darkfield microscopy involves measuring red blood cell (RBC) diameter to assess for microcytosis or macrocytosis. The reference range for normal RBC diameter currently used in fresh capillary blood analysis by darkfield microscopy (FCB-DM) is the same as that used in haematology, i.e. 6.2–8.2 μm. However, the haematology reference range for RBC diameter refers to stained and dried blood specimens. Evidence from early research suggests that fresh RBCs are larger than stained and dried RBCs. The following article presents methodology and findings for the objective measurement of RBC diameter using the FCB-DM technique.

Methods: FCB-DM screenings of 14 consenting participants were photographed to permit a quantitative analysis of RBC size and morphology. Participants also provided a venous blood sample for pathology testing, which included markers for RBC size. The researcher was blinded to the pathology results until all FCB-DM data analysis was complete.

Results: All participants had a normal reference range for all pathology markers tested. The mean RBC diameter, determined by FCB-DM, was 8.51 μm (s = 0.161, s^2 = 0.026, range = ±0.656) and the mode was 8.55 μm.

Conclusion: These findings support evidence from early studies of RBC diameter, that fresh blood cells observed with darkfield microscopy are approximately 1.3 μm larger than dried and stained RBCs. Further research using a larger sample size is recommended.

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What is currently known?

- Currently, the measurement of RBC diameter using the FCB-DM technique refers to previously established haematological indices for stained and dried blood samples assessed as part of a peripheral blood smear. Although visual estimation of RBC size is now a dated practice in haematology due to the development of automated cell measurement, these haematological indices of RBC size are relevant for the purposes of FCB-DM that is a visual, point-of-care screening tool.
- Historic research suggests that the average diameter of fresh RBCs from unstained blood samples is larger than those seen in dried, stained blood samples.
- There is a paucity of research on the FCB-DM technique and no published articles detailing explicitly the sampling and analysis of blood using this technique.

What this paper adds

- FCB-DM sampling and analysis procedure currently taught and used in clinical practice.
- Findings from an objective analysis of RBC diameter in a sample of 14 participants.
- An indication that the FCB-DM method of measuring RBCs currently being taught and practiced needs to be re-evaluated to represent current data indicating greater cell size with darkfield microscopy.

1. Introduction

Fresh capillary blood analysis using darkfield microscopy (FCB-DM), also referred to as 'live blood analysis', is a technique currently used by clinicians as a point-of-care screening tool to assess for haematological status [1]. Using the FCB-DM technique, clinicians can assess blood cell morphology and dynamics without the need of staining or drying procedures – a significant advantage for point-of-care testing. As part of a FCB-DM screening, practitioners assess the size of red blood cells (RBCs) to screen for macrocytosis and microcytosis. As a screening tool, FCB-DM

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does not aim to be diagnostic and therefore is not intended as a substitute for cytometric pathology testing. The potential advantages of using FCB-DM's visual screening method is that several health aberrations can be screened for simultaneously and immediately in a clinical setting. Clinician could also utilise FCB-DM in follow up consultation to check treatment efficacy. There is a paucity of research investigating the FCB-DM technique and none to date that specifically addresses the measurement of RBC size. Therefore FCB-DM training manuals refer to haematology literature [2] for reference values, including the reference range of a normal RBC diameter (6.2–8.2 μm with an average RBC diameter of 7.2 μm). However, the RBC diameter reference range used in haematology refers to dried and stained RBC specimens. Findings from early haematological research concluded that the diameter of a fresh RBC is larger than a dried RBC [3,4].

The first direct measurement of RBC diameter was reported in 1674 by van Leeuwenhoek, who with his rudimentary microscope made an estimate of RBC size to be 8.3 μm [5]. It was first noted by Ponder and Miller [6] in their early studies of RBC morphology that the diameter of a RBC once dried and stained was considerably smaller than RBCs in plasma and according to their observations the actual size of a RBC suspended in plasma was 8.8 μm. There were six subjects in the trial, all male and apparently healthy. For comparison the authors also prepared stained and dried blood smears. The RBCs in the dried samples were found to be approximately 1.0 μm smaller in diameter. In a later study, Ponder and Saslow [3] revised this estimate of the average RBC diameter to be 8.5 μm ± 0.5 and the shrinkage of dried RBCs to be 8–16% [7]. The diameter of fresh RBCs was also investigated by Houchin et al. [8], who found the mean diameter of an unstained and undried RBC to be 8.28 μm ± 0.6, however, their study was specifically analysing cells in a state of rouleaux, which can distort red cell shape, and no reference was made as to whether the blood was from healthy subjects or not. The most recent relevant study by Westerman et al. [4] found the mean diameter of a fresh RBC taken from healthy individuals to be similar to Ponder and Saslow's at 8.56 μm ± 0.21.

To improve the precision and accuracy of the FCB-DM technique, particularly in regard to future research, it is essential that a correct reference range be established for the FCB-DM assessment of RBC diameter. It is expected that the diameter of RBCs measured in this study will be closer to the size reported by Ponder and Saslow [3] and Westerman et al. [4] of 8.5–8.6 μm.

2. Materials and methods

2.1. Participants

Participants underwent FCB-DM screening and pathology testing for the markers shown in Table 1. The inclusion criterion for this study was that participants should have normal levels of the tested pathology markers and 14 participants (3 males and 11 females) were included. Data were collected over a period of one month at Southern Cross University (SCU) with informed consent obtained from all participants. The research was approved by the Human Research Ethics Committee at SCU (approval number ECN-11-247).

Table 1
Pathology tests performed and their reference ranges. All pathology results for participants in this study were within the normal range.

Pathology marker	Reference range
Serum ferritin	15–165 μg/L
Mean cell volume	80–98 fL
Haemoglobin	120–165 g/L
Methylmalonic acid	<0.370 μmol/L
Homocysteine	<15.0 μmol/L
RBC folate	>565 nmol/L
Thyroid stimulating hormone	<4.0 mU/L
C-reactive protein	<5.0 mg/L
Erytrhocyte sedimentation rate	<20.0 mm/h
Liver function tests	Standard laboratory references[a]

[a] Standard references used by QML Pathology.

2.2. Fresh capillary blood sampling procedure for darkfield microscopy

Capillary blood samples from participants were collected immediately before the FCB-DM screening. After swabbing with an alcohol wipe, the third or fourth finger was pierced with a retractable, sterile lancet (0.81 mm, Owen Mumford). The skin around the prick site was gently stretched to encourage a blood droplet to appear. The first drop was lightly wiped away with a Kimwipe (Kimberley Clarke) to avoid collecting damaged cells caused by the lancet. Two slides (Menzel-Gläser 1 mm) were then prepared so that the slide with the larger working zone could be selected for data collection. The first slide was carefully touched to the next drop of blood without making contact with the skin. A cover slip (Deckgläser 22 × 40 mm) was applied to the slide within 10 seconds of the drop of blood being collected to avoid prolonged exposure to air. A second slide was prepared by first lightly wiping away any blood from the fingertip and then sampling another drop of blood. A timer was started from the moment the second cover slip was in place. Blood glass interactions will initiate coagulation of RBCs and fibrin formation within 30 min of blood contacting the slide, distorting the appearance of RBCS [9]. Therefore, all RBC data was collected within ten minutes of taking the fingertip blood to ensure optimal clarity of RBCs. Immediately after the FCB-DM screening or at the same time the next day, participants provided a sample of venous blood for pathology testing, performed by Queensland Medical Laboratories (QML). The researcher was blinded to the results of pathology tests until all FCB-DM analysis was complete.

2.3. Darkfield microscopy and measurement of RBC diameter

The measurement of RBC diameter was assessed using a Prism Optical Pro 2050 (Prism Optical, China) microscope with a darkfield cardioid, oil immersion condenser. A Watec 221S camera system (Watec, Japan) with low colour and 768 × 572 PAL output was fitted to the trinocular head, and connected to a computer. For each blood screening, twenty consecutive fields of vision of the working zone of the slide were photographed at 400x magnification, using oil immersion. The photographs were captured using Metascreen™ software (Health World, Australia) and analysed using ImageJ software [10]. The microscope was calibrated with the ImageJ software as follows:

- A slide micrometre with 0.01 mm intervals was photographed at 400× magnification using the same darkfield microscope used for data collection.
- The slide micrometre image was opened with ImageJ software and using the line measuring tool, a straight line measuring 100 μm was selected and a scale set to the number of pixels per μm on the slide micrometre. The scale determined was 4.4 pixels: 1 μm.

The accurate measurement of RBC diameter using darkfield microscopy also required the correct strength of light to illuminate the specimen. It was observed during preliminary calibrations that the width of RBC membranes viewed under darkfield conditions increased from approximately 0.6 μm to 1.2 μm when the light source illuminating the specimen was too strong, as demonstrated in Fig. 1. To achieve optimal clarity of membrane borders, it was

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