Quantification Of Very Low Concentrations Of Leukocyte Suspensions In Vitro By High-Frequency Ultrasound.

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1 **ABSTRACT**

2 Accurate measurement of very low cerebrospinal fluid (CSF) white blood cell
3 (WBC) concentration is key to the diagnosis of bacterial meningitis, lethal if not
4 promptly treated. Here we show that High Frequency Ultrasound (HFUS) can
5 detect CSF WBC *in vitro* in concentrations relevant to meningitis diagnosis with a
6 much finer precision than gold standard manual counting in a Fuchs-Rosenthal
7 chamber. WBC concentrations in a mock CSF model, in the range 0-50 WBC/ μ L,
8 have been tested and compared to gold standard ground truth. In this range,
9 excellent agreement (Cohen's kappa $k=0.78-90$) was observed between HFUS
10 and the gold standard method. The presented experimental setup allowed us to
11 detect WBC concentrations as low as 2 cells/ μ L. HFUS shows promise as a low-
12 cost, reliable and automated technology to measure very low CSF WBC
13 concentrations for the diagnosis of early meningitis.

14

15 **KEYWORDS:** cerebrospinal fluid, WBCs, cell count, high frequency ultrasound,
16 meningitis, cytometry.

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1 INTRODUCTION

2 Accurate determination of the leukocyte (WBC) concentration of
3 cerebrospinal fluid (CSF) samples is clinically relevant in the diagnosis of
4 meningitis (Kestenbaum et al. 2011; Manning et al. 2014). CSF is normally
5 acellular and the presence of low (5-25 WBC/ μ L (Edwards et al. 2013;
6 Greenberg et al. 2011)) yet abnormal level of WBC raises clinical suspicion of
7 meningeal infection. In the case of bacterial meningitis (BM), early detection and
8 immediate treatment are essential to improve prognosis and prevent lethal
9 effects (Ishimine 2006). Currently, CSF samples are extracted through a lumbar
10 puncture (LP) and WBC concentration can be obtained within an hour. If
11 diagnostic CSF WBC levels are exceeded and meningeal infection is suspected,
12 treatment will be initiated. Because the blood brain barrier (BBB) becomes less
13 permeable with age, diagnostic levels for CSF WBC concentration in meningitis
14 are higher for patients younger than 29 days (>20 -25 WBC/ μ L) than for patients
15 between 30-90 days (>9 WBC/ μ L) and older than 90 days (>5 WBC/ μ L) (Army
16 2003; Edwards et al. 2013; Greenberg et al. 2011).

17

18 The clinical gold standard procedure to measure such low concentrations
19 in CSF is the manual count of LP samples with a Fuchs-Rosenthal cell counting
20 chamber, which has associated measurement variability 30-50% at
21 concentrations below 50 cells/ μ L, is operator dependent and time consuming (De
22 Jonge et al. 2006; Kleine et al. 2012; Ngoyi et al. 2013; Zimmermann et al.
23 2011). Alternatively, automated cell counters are mostly designed for

1 haematology analysis and hence, rely on a number of cell counts higher than
2 those in CSF samples to provide a statistically confident measurement (De
3 Jonge et al. 2006; Ongena et al. 2010; Zimmermann et al. 2011). High
4 Frequency Ultrasound (HFUS) has successfully been applied for cell
5 characterization from the ultrasonic backscatter of individual cells (Baddour and
6 Kolios 2006a). This technique has also been used to measure the integrated
7 backscatter coefficient (BSC) from cells as an estimation of cell concentration in
8 fluid samples. To compute BSC the ultrasound signals centred at the acoustic
9 focus are filtered with a Hamming window to suppress spectral lobes. Then the
10 Fourier transform is computed and squared to obtain the backscatter power
11 spectrum $S(f)$, which can be directly related to pressure intensities (Baddour et
12 al. 2005). To calculate the absolute backscatter power spectrum, $S(f)$ is
13 normalized ($S_{\text{NORM}}(f)$) by dividing it by a reference spectrum ($S_{\text{REF}}(f)$) of the echo
14 from a planar steel, brass or crystal reflector also centred at the focus to remove
15 system and transducer transfer function (Baddour and Kolios 2006; Elvira et al.
16 2016; Mercado et al. 2014). Nevertheless, to show relative differences between
17 measurements obtained with the same equipment, normalization is not
18 necessary and has not been performed herein. When the medium used in the
19 experiment is different than that used to obtain the reference spectrum, the
20 frequency-dependent attenuation coefficient of the medium used in the
21 experiment is applied to $S_{\text{NORM}}(f)$ (Mercado et al. 2014; O'Donnell and Miller
22 1981). When a water-like medium is used, medium attenuation is negligible and
23 may not be factored into the calculations (Kolios et al. 2004). While the frequency

backscatter response allows modelling and characterization of the acoustic response of individual cells (Baddour et al. 2002), quantification of cell concentration is provided by the integrated $S_{\text{NORM}}(f)$ over the -6dB bandwidth of the pulse. We refer to this integrated coefficient as the BSC power, although is named differently in other studies (Baddour and Kolios 2006; Mercado et al. 2014; O'Donnell and Miller 1981; Vlad et al. 2008). Other authors (Baddour and Kolios 2006; Kolios et al. 2004; Tunis et al. 2005) used BSC to measure cell solution samples at concentrations above 50 cells/ μL with a precision between tens to hundreds of cells/ μL . A later work (Mercado et al. 2014) focusing on backscatter cell concentration measurement in engineered tissue showed a detection limit of 50 cells/ μL . However, the authors aimed at providing a tissue characterization metric of the concentration of 10- μm cells in tissue, not fluids. To the best of our knowledge, the 0-50 cells/ μL range has not been quantified with HFUS and is relevant in the diagnosis of early-stage meningitis.

In this paper, the capability of HFUS to measure WBC concentrations in the range 0-50 WBC/ μL was explored. Series of samples of WBC concentrations in serum saline at 0.5 g/L plasma protein concentration were made to mimic healthy and early-infected CSF. These samples were manually characterized at the routine analysis laboratory in University Hospital Clínico San Carlos (Madrid, Spain) using a Fuchs Rosenthal chamber. The coefficient of determination provided the degree of linear relationship between the methods. Agreement was measured with the Cohen's kappa coefficient for the different CSF WBC

1 concentrations for meningitis diagnosis. A Bland-Altman analysis determined the
2 bias and 95% confidence interval of the paired-measurements differences
3 between methods.

5 **MATERIAL AND METHODS**

6 *Preparation of cell solutions*

7 Blood samples from 20 patients with leukocytosis arriving at the routine
8 analysis laboratory in University Hospital Clínico San Carlos were collected in
9 EDTA-K3 tubes (BD Vacutainer®, BD, Franklin Lakes, New Jersey, USA).
10 Approval of the study was obtained from hospital Ethics Committee and all
11 volunteers gave their informed consent. The tubes were centrifuged at 400 g for
12 15 minutes and between 1 and to 2 mL of plasma from each EDTA tube were
13 used to create a pool of plasma. A method based on the modification of
14 (Weichselbaum 1946) was used to measure protein pool in the pool with an
15 AU5800 analyzer (Beckman-Coulter, Pasadena, California, USA). Saline serum
16 (B. Braun Medical, Melsungen, Germany) was added to obtain a final solution
17 volume of 50 mL at 0.5 g/L protein level. This protein matrix constituted the
18 healthy model of CSF. From each EDTA tube, 1 mL of buffy coat was pipetted
19 out with a Pasteur pipette (Copan, Brescia, Italy) and transferred to a Wintrobe
20 tube (Fisher Scientific, Madrid, Spain). Ten Wintrobe tubes were then centrifuged
21 at 400 g for 15 minutes and buffy coat components were separated in four
22 distinct layers: plasma only, platelets enriched plasma, a leuko-platelet layer and
23 an erythrocytes layer. Plasma was discarded and the platelet-enriched plasma

1 was saved to assess the impact of platelets in the ultrasound measurement. For
2 each tube, the leuko-platelet layer was pipetted out and diluted in 1 mL of the
3 protein matrix. An additional centrifugation at low speed (40 g for 5 minutes) was
4 performed to separate the majority of platelets. Supernatant was eliminated and
5 the pellet was re-suspended with 1 ml of the protein matrix. From this stock
6 suspension, cell dilutions were prepared by diluting high-concentration samples
7 that had been previously measured by a haematology system (Advia 120,
8 Siemens Healthcare, Germany) to obtain concentrations in the range 0-50
9 WBCs/ μ L. The measurement error of these systems for concentrations above
10 100 cells/ μ L is 5-10% (De Jonge et al. 2006; Ongena et al. 2010; Zimmermann et
11 al. 2011). Therefore, since diluted samples were prepared in a controlled
12 manner, their approximate concentration was known and manual counting could
13 next validate their concentration, which determined the ground truth data for the
14 experiments. Total and differential manual cell counts were performed in a
15 Fuchs-Rosenthal chamber (Paul Marienfeld GmbH & Co., Lauda-Königshofen,
16 Germany). Finally, 24 cell solution samples were prepared with an average
17 proportion of 77% WBCs and 23% erythrocytes. Although, an erythrocyte
18 concentration of 23% is not representative of CSF in patients suspected for or
19 with meningitis, the ultrasound method showed an imperceptible response to
20 erythrocyte concentration in this cell concentration range, as demonstrated in the
21 discussion section. Within the WBC population, the polymorphonuclear (PMN) to
22 mononuclear (MN) cell proportion was 1/4 in 6 samples, 1/9 in 7 samples and

9/10 in 8 samples. In three out of the 24 cell solution samples no cells were added and the cell count was zero.

Ultrasound data acquisition and analysis

Cell solution samples were scanned with ultrasound using a single element transducer (V3320, Olympus, Waltham, MA, USA). The transducer centre frequency was 75 MHz with a -6dB bandwidth ranging from 42.5MHz to 101MHz, a focal point located at 12.5 mm and an f-number of 2. The transducer was excited with a Panametrics 5900PR pulser (Olympus, Waltham, MA, USA) and connected to a Picoscope 6402D oscilloscope (Pico Technology, Cambridgeshire, United Kingdom). 1500 sequential signals centred in the focus were acquired every 0.2 s at a sampling rate of 1.25 GHz and stored in a workstation for post-processing. Cell solution samples were put in direct contact with the transducer and contained in a specifically constructed PVC lid that was coiled onto the transducer. Cell solution samples were pipetted in and out of the lid cavity through a 6 mm aperture (Fig. 1). According to a cell viability analysis performed at the hospital, samples were kept at 4 °C and measured within three hours from elaboration to guarantee a minimum 90 % cell sample viability at the start of the ultrasound measurement. A 450 µL sample volume was used to guarantee immersion of the acoustic focus in the sample. BSC was computed from the signals using the method described in (Vlad et al. 2008) and compared to the Fuchs-Rosenthal counts. The ultrasound data was not normalized to any reference signal because the purpose of the work was to measure BSC

differences between cell solution samples at different concentrations rather than analysing absolute cell BSC measurements. From now on we will refer to the ultrasound method as BSC method. Ultimately, the BSC measurement variability depends on: 1) the cell concentration variability between the elaborated sample and the actual 450 μ L subsample extracted from it for measurement and 2) the variability at such low concentrations in the proportion of cells crossing the focal zone with respect to cell concentration (Elvira et al. 2016). To minimize the effect of these two factors in the BSC measurement, two 450 μ L samples were measured for each cell solution sample and the average of the two measurements constituted the BSC measurement.

Statistical analysis

To validate the WBC counts on the BSC method, the coefficient of determination (R^2) and the linear regression model was calculated. This model was next inverted to obtain an estimate of the WBC concentration from BSC. Bias between the methods was analysed using a Bland-Altman difference plot (Bland and Altman 1986). Agreement between the methods to determine CSF condition based on CSF cell concentration measurements was calculated by means of the Cohen's kappa coefficient (κ) (Cohen 1960). WBC concentration measurements below/above meningitis CSF concentration levels 5, 9 and 20 WBC/ μ L (Army 2003; Edwards et al. 2013) were used to classify cell solution samples as normal/infected. Data processing and all statistical analyses were performed using Matlab (R2014b, The MathWorks Inc., Natick, Massachusetts).

1

2 RESULTS

3 A total of 24 cell solution samples in the range 0-61 WBC/ μ L were
4 measured in a Fuchs-Rosenthal chamber and with the BSC method. Fig. 2
5 shows the linear trend that relates BSC (left axis) to the gold standard WBC
6 count. Results showed a linear agreement (R^2 : 0.95) of the backscatter signal
7 with increasing WBC concentration.

8

9 The linear model that predicted with a 95% confidence level the amount of
10 BSC for a certain WBC concentration as measured in a Fuchs-Rosenthal
11 chamber (H) is formulated as

12

$$13 \quad \text{BSC} = p1 \times H + p2 \quad (1)$$

14

15 With $p1 = 7.84 \times 10^{-5}$ (slope coefficient) and $p2 = 5.79 \times 10^{-3}$ (coordinate axis
16 cut value). In order to measure the agreement with the gold standard, an
17 estimate of WBC concentration (E_{WBC}) from BSC is needed. This estimate of
18 WBC concentration was obtained by inverting the linear model in (1):
19 $E_{WBC} = (\text{BSC} - p2) / p1$. The scatter plot of E_{WBC} in relation to the gold standard is
20 shown in Fig. 2 (right axis). The Cohen's kappa coefficient showed almost perfect
21 agreement between methods in determining whether samples WBC
22 concentration exceeded two clinically diagnostic values for meningitis: 9 WBC/ μ L
23 (Cohen $\kappa = 0.81$; 95% CI, 0.57–1) and 20 WBC/ μ L (Cohen $\kappa = 0.90$; 95% CI,

0.72–1) and substantial agreement was shown for 5 WBC/ μ L (Cohen κ = 0.78; 95% CI, 0.49–1).

The Bland-Altman difference plot analysis demonstrated an average difference, i.e. bias, between the Fuchs-Rosenthal chamber and BSC measurements <0.01 WBC/ μ L (Fig. 3). The 95% limits of agreement between paired measurements in the 0-61 WBC/ μ L range were -8.11 to 8.11 WBC/ μ L.

When restricting the analysis to the range of WBC concentration values in the CSF with diagnostic value in meningitis (0,25] WBC/ μ L, the Bland-Altman difference analysis (Fig. 4) showed a bias between the methods of -1.55 WBC/ μ L (95% CI: -7.93-4.83 WBC/ μ L). For healthy cases, where no WBCs are present in the CSF samples according to the Fuchs-Rosenthal chamber, our BSC-based model produces negative estimates (Fig. 2) avoiding the possibility to misdiagnose these cases as false positives.

DISCUSSION

The capabilities of HFUS to quantify very low (0-50 cells/ μ L) concentrations of WBCs in a CSF-like medium as clinically relevant information in meningitis diagnosis were analyzed. An excellent (R^2 : 0.95) linear agreement was observed between the WBC measurements with the gold standard Fuchs-Rosenthal chamber and the BSC method. Since the samples were elaborated

1 from human blood and perfect WBC isolation was not possible, platelets and
2 erythrocytes were present in all the samples. In a separate experiment, samples
3 containing only platelets at different dilutions in plasma were measured. With this
4 experiment it was confirmed that no detectable echo coming from platelets could
5 be obtained with the experimental set-up used, therefore removing the possibility
6 of confounding measurements introduced by platelet contamination: In all cases,
7 BSC values from platelet-only samples did not surpass the noise level measured
8 from plasma-only samples. Erythrocyte contribution to BSC results was also not
9 substantial. Fundamentally, erythrocytes have a disc shape of approximately 2 x
10 7 μm and no nuclei (Woessner, S., Lafuente and Florensa 2006). Because of this
11 disc shape, their backscattered signal is dependent on the incidence angle of the
12 wave (Kuo and Shung 1994). As a result, incident waves impinging on the long-
13 axis surface of erythrocytes at non-orthogonal angles will reflect weaker
14 backscatter signals. The absence of nuclei in erythrocytes, as a major cell
15 composition difference with WBCs, also explains their minor contribution to BSC.
16 In (Kolios et al. 2004) the BSC contribution of cells with different nucleus-to-cell
17 volume ratios in suspension and in pellet was studied. The authors concluded
18 that the nucleus is strongly contributing to BSC and that a larger effective scatter
19 size of cells in suspension also increases BSC. In addition to this, Vlad et al.
20 (Vlad et al. 2008) showed that nuclei composition has also an effect in the BSC
21 signal. The authors observed that nuclei changes associated with cell death
22 increased BSC by more than 10 dB. In the present work, when the measured
23 BSC was related not only to the WBC concentration in the samples but to their

1 joint WBC and erythrocyte concentration, the coefficient of determination
2 decreased (R^2 : 0.91). These results are aligned with previous findings. A clear
3 example of the questionable impact of erythrocytes in the sample was also found
4 on a sample producing similar BSC, with exactly the same WBC concentration
5 and an erythrocyte concentration difference of 19 erythrocytes/ μ L (Table 1,
6 shaded cells). Finally, the step increase in BSC from samples with no cells to
7 samples with 2 WBC/ μ L was not well represented by the linear model relating
8 BSC and manual measurements with a Fuchs-Rosenthal counting chamber. For
9 this reason, when inverting the model to estimate WBC concentration from BSC,
10 negative estimated concentrations were obtained for samples with no cells.

11
12 BSC depends on the wavelength-to-cell size factor, i.e. ka (Baddour et al.
13 2005). The characterized samples contained a wide percentages range of PMN
14 and MN cells. The PMN-to-MN ratio was 1/4 in 6 samples, 1/9 in 7 samples and
15 9/10 in 8 samples. PMNs are 12-14 μ m in size whereas MNs can be classified
16 into monocytes (15-30 μ m) and lymphocytes (8-10 μ m) (Woessner, S., Lafuente
17 and Florensa 2006). However, this mixture of PMNs and MNs had no significant
18 effect in the observed linearity between methods. Consequently, our set up was
19 mostly sensitive to WBCs, larger in dimensions and in volume than erythrocytes
20 and platelets. This would be in agreement with the study performed by Kolios et
21 al. (Kolios et al. 2004) with acute myeloma cells (10 μ m) and prostate cancer
22 cells (25 μ m) hypothesizing that, when cells are suspended in fluid, cell volume
23 was the major contributor to BSC.

1

2 Excellent statistical agreement between the methods resulted from
3 analysis of the Cohen's kappa coefficient (Cohen's $\kappa=0.78-0.90$ for all CSF
4 diagnostic levels) and of the Bland-Altman plot (Fig. 4), which showed a bias
5 between the methods of -1.55 WBCs/ μL and a standard deviation of 3.26
6 WBC/ μL between paired-measurement differences. Assuming equivalence
7 between methods, the coefficient of variation (CV) was computed from the paired
8 measurements of the two methods. In the $2-30$ WBC/ μL range 94% ($16/17$) of
9 the measurements produced $\text{CV} \leq 27\%$, superior results than the $\text{CV}=30-50\%$
10 reported for repeated manual counting of CSF samples in the Fuchs-Rosenthal
11 chamber at concentrations below 50 WBC/ μL (De Jonge et al. 2006; Kleine et al.
12 2012; Ngoyi et al. 2013; Zimmermann et al. 2011).

13

14 A BSC method is presented as low-cost, tabletop, and accurate solution to
15 measure CSF WBC concentration in the $0-50$ cells/ μL range where the
16 diagnostic levels in early stage meningitis for all ages are comprised and other
17 technologies cannot reliably perform. The CV results obtained present the BSC
18 method as a promising automated and more accurate alternative to manual
19 counting in a Fuchs-Rosenthal chamber of CSF WBC concentration.

20

21 The presented experimental setup was shown to be a sensitive system to
22 very low WBC concentrations requiring very low sample volume ($450 \mu\text{L}$), as
23 opposed to other studies of the backscatter response of individual cells where

the setup involves immersion of the transducer in a larger sample (Baddour and Kolios 2006b; Kolios et al. 2004; Tunis et al. 2005). Moreover, using a transducer with a shorter focus could further reduce the minimum sample volume. This would be an intermediate step towards a system designed for sample reusability. In such system the sample could be measured by means of ultrasound while confined in a container or syringe made of material with low acoustic attenuation. This is an important consideration in CSF samples from pre-terms and newborns when it is not uncommon that too little sample is drawn to perform the biochemical, cytological and culture analysis. In this work, the first results showing a linearly increasing relationship between BSC to WBC suspensions below 50 cells/ μ L are presented.

Future work will assess the capabilities of the BSC method to differentiate between different WBC types. On views of its applicability in the clinical setting, a shorter focal distance will be used to reduce the required CSF sample volume.

CONCLUSIONS

The current study shows the feasibility of High Frequency Ultrasound (HFUS) to measure WBC concentrations in cerebrospinal fluid (CSF)-like medium in the diagnostic range for meningitis. The results obtained showed excellent agreement (Cohen $k=0.78-90$, mean paired-measurement differences - 1.55 WBC/ μ L and 95% CI: -7.93 to 4.83 WBC/ μ L) with gold standard manual counting in a Fuchs-Rosenthal chamber in the 0-30 WBC/ μ L.

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1 REFERENCES

- 2 Army T. Cerebrospinal Fluid Analysis. *Am Fam Physician* 2003;68:1103–1108.
- 3 Baddour RE, Kolios MC. The fluid and elastic nature of nucleated cells:
4 Implications from the cellular backscatter response. *J Acoust Soc Am*
5 2006a;121:16–22.
- 6 Baddour RE, Kolios MC. Investigating the Effect of Cell Size on the Backscatter
7 from Suspensions of Varying Volume Fractions. *IEEE Ultrason Symp* 2006b.
8 pp. 637–640.
- 9 Baddour RE, Sherar MD, Czarnota GJ, Hunt JW, Taggart L, Giles A, Farnoud
10 NR, Kolios MC. High frequency ultrasound imaging of changes in cell
11 structure including apoptosis. *2002 IEEE Ultrason Symp 2002 Proceedings*
12 2002;2:1639–1644.
- 13 Baddour RE, Sherar MD, Hunt JW, Czarnota GJ, Kolios MC. High-frequency
14 ultrasound scattering from microspheres and single cells. *J Acoust Soc Am*
15 2005;117:934–943.
- 16 Bland J, Altman D. Statistical methods for assessing agreement between two
17 methods of clinical measurement. *Lancet* 1986;1:307–310.
- 18 Cohen J. A coefficient of agreement for nominal scales. *Educ Psychol Meas*
19 1960;20:37–46.
- 20 De Jonge R, Brouwer R, Van Rijn M, Van Acker B a C, Otten HJ a M, Lindemans
21 J. Automated analysis of pleural fluid total and differential leukocyte counts
22 with the Sysmex XE-2100. *Clin Chem Lab Med* 2006;44:1367–1371.
- 23 Edwards MS, Nordli DR, Editor D, Torchia MM. Clinical features and diagnosis of
24 acute bacterial meningitis in children older than one month of age. In: Basow
25 DS, ed. *UpToDate* Waltham: UpToDate, 2013. pp. 1–19.
- 26 Elvira L, Vera P, Cañadas FJ, Shukla SK, Montero F. Concentration
27 measurement of yeast suspensions using high frequency ultrasound
28 backscattering. *Ultrasonics Elsevier B.V.*, 2016;64:151–161.
- 29 Greenberg RG, Benjamin DK, Cohen-Wolkowicz M, Clark RH, Cotten CM,
30 Laughon M, Smith PB. Repeat lumbar punctures in infants with meningitis in
31 the neonatal intensive care unit. *J Perinatol Nature Publishing Group*,
32 2011;31:425–9.
- 33 Haïat G, Berti R, Galaz B, Taulier N, Amman J-J, Urbach W. Two-dimensional
34 simulation of linear wave propagation in a suspension of polymeric
35 microcapsules used as ultrasound contrast agents. *J Acoust Soc Am*
36 2011;129:1642–1652.
- 37 Ishimine P. Fever without source in children 0 to 36 months of age. *Pediatr Clin*
38 *North Am* 2006;53:167–94.
- 39 Kestenbaum LA, Ebberson J, Zorc JJ, Hodinka RL, Shah SS. Determining
40 cerebrospinal fluid white blood cell count reference values in neonates and
41 young infants. *Pediatrics* 2011;125:257–264.

1 Kleiue TO, Nebe CT, Löwer C, Geilenkeuser WJ, Dorn-Beineke A. Cell analysis
2 in cerebrospinal fluid (CSF) using Sysmex ® hematology analyzers XT-4000i
3 and XE-5000: Evaluation with CSF controls of the Joint German Society for
4 Clinical Chemistry and Laboratory Medicine (DGKL). Cytom Part A 2012;81
5 A:255–264.

6 Kolios M, Czarnota G, Worthington A, Giles A, Tunis A. Towards Understanding
7 the Nature of High Frequency Ultrasound Backscatter from Cells and
8 Tissues : an Investigation of Backscatter Power Spectra from Different
9 Concentrations of Cells of Different Sizes. IEEE Ultrason Symp 2004;606–
10 609.

11 Kuo IY, Shung KK. High frequency ultrasonic backscatter from erythrocyte
12 suspension. IEEE Trans Biomed Eng 1994;41:29–34.

13 Manning L, Laman M, Mare T, Hwaiwhanje I, Siba P, Davis TME. Accuracy of
14 cerebrospinal leucocyte count, protein and culture for the diagnosis of acute
15 bacterial meningitis: a comparative study using Bayesian latent class
16 analysis. Trop Med Int Health 2014;19:1520–4.

17 Mercado KP, Helguera M, Hocking DC, Dalecki D. Estimating cell concentration
18 in three-dimensional engineered tissues using high frequency quantitative
19 ultrasound. Ann Biomed Eng 2014;42:1292–1304.

20 Ngoyi DM, Menten J, Pyana PP, Büscher P, Lejon V. Stage determination in
21 sleeping sickness: Comparison of two cell counting and two parasite
22 detection techniques. Trop Med Int Heal 2013;18:778–782.

23 O'Donnell M, Miller JG. Quantitative Broadband Ultrasonic Backscatter: an
24 Approach To Nondestructive Evaluation in Acoustically Inhomogeneous
25 Materials. J Appl Phys 1981;52:1056–1065.

26 Ongena K, Das C, Smith JL, Gil S, Johnston G. Determining Cell Number During
27 Cell Culture using the Scepter Cell Counter. J Vis Exp 2010;1–5.

28 Tunis AS, Baddour RE, Czarnota GJ, Giles A, Worthington a. E, Sherar MD,
29 Kolios MC. Using high frequency ultrasound envelope statistics to determine
30 scatterer number density in dilute cell solutions. Proc - IEEE Ultrason Symp
31 2005;2:878–881.

32 Vlad RM, Alajez NM, Giles A, Kolios MC, Czarnota GJ. Quantitative ultrasound
33 characterization of cancer radiotherapy effects in vitro. Int J Radiat Oncol
34 Biol Physiscs 2008;72:1236–1243.

35 Weichselbaum T. An accurate and rapid method for the determination of proteins
36 in small amounts of blood serum and plasma. Am J Clin Pathol 1946;16:40–
37 9.

38 Woessner, S., Lafuente R, Florensa L. La citología óptica en el diagnóstico
39 hematológico. 5th ed. Medici, 2006.

40 Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann a. Automated
41 vs. manual cerebrospinal fluid cell counts: A work and cost analysis
42 comparing the Sysmex XE-5000 and the Fuchs-Rosenthal manual counting

- chamber. *Int J Lab Hematol* 2011;33:629–637.
- Army T. Cerebrospinal Fluid Analysis. *Am Fam Physician* 2003;68:1103–1108.
- Baddour RE, Kolios MC. The fluid and elastic nature of nucleated cells: Implications from the cellular backscatter response. *J Acoust Soc Am* 2006a;121:16–22.
- Baddour RE, Kolios MC. Investigating the Effect of Cell Size on the Backscatter from Suspensions of Varying Volume Fractions. *IEEE Ultrason Symp* 2006b. pp. 637–640.
- Baddour RE, Sherar MD, Czarnota GJ, Hunt JW, Taggart L, Giles A, Farnoud NR, Kolios MC. High frequency ultrasound imaging of changes in cell structure including apoptosis. *2002 IEEE Ultrason Symp 2002 Proceedings* 2002;2:1639–1644.
- Baddour RE, Sherar MD, Hunt JW, Czarnota GJ, Kolios MC. High-frequency ultrasound scattering from microspheres and single cells. *J Acoust Soc Am* 2005;117:934–943.
- Bland J, Altman D. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–310.
- Cohen J. A coefficient of agreement for nominal scales. *Educ Psychol Meas* 1960;20:37–46.
- De Jonge R, Brouwer R, Van Rijn M, Van Acker B a C, Otten HJ a M, Lindemans J. Automated analysis of pleural fluid total and differential leukocyte counts with the Sysmex XE-2100. *Clin Chem Lab Med* 2006;44:1367–1371.
- Edwards MS, Nordli DR, Editor D, Torchia MM. Clinical features and diagnosis of acute bacterial meningitis in children older than one month of age. In: Basow DS, ed. *UpToDate Waltham: UpToDate*, 2013. pp. 1–19.
- Elvira L, Vera P, Cañadas FJ, Shukla SK, Montero F. Concentration measurement of yeast suspensions using high frequency ultrasound backscattering. *Ultrasonics Elsevier B.V.*, 2016;64:151–161.
- Greenberg RG, Benjamin DK, Cohen-Wolkowicz M, Clark RH, Cotten CM, Laughon M, Smith PB. Repeat lumbar punctures in infants with meningitis in the neonatal intensive care unit. *J Perinatol Nature Publishing Group*, 2011;31:425–9.
- Haïat G, Berti R, Galaz B, Taulier N, Amman J-J, Urbach W. Two-dimensional simulation of linear wave propagation in a suspension of polymeric microcapsules used as ultrasound contrast agents. *J Acoust Soc Am* 2011;129:1642–1652.
- Ishimine P. Fever without source in children 0 to 36 months of age. *Pediatr Clin North Am* 2006;53:167–94.
- Kestenbaum LA, Ebberson J, Zorc JJ, Hodinka RL, Shah SS. Determining cerebrospinal fluid white blood cell count reference values in neonates and young infants. *Pediatrics* 2011;125:257–264.

1 Kleiue TO, Nebe CT, Löwer C, Geilenkeuser WJ, Dorn-Beineke A. Cell analysis
2 in cerebrospinal fluid (CSF) using Sysmex ® hematology analyzers XT-4000i
3 and XE-5000: Evaluation with CSF controls of the Joint German Society for
4 Clinical Chemistry and Laboratory Medicine (DGKL). Cytom Part A 2012;81
5 A:255–264.

6 Kolios M, Czarnota G, Worthington A, Giles A, Tunis A. Towards Understanding
7 the Nature of High Frequency Ultrasound Backscatter from Cells and
8 Tissues : an Investigation of Backscatter Power Spectra from Different
9 Concentrations of Cells of Different Sizes. IEEE Ultrason Symp 2004;606–
10 609.

11 Kuo IY, Shung KK. High frequency ultrasonic backscatter from erythrocyte
12 suspension. IEEE Trans Biomed Eng 1994;41:29–34.

13 Manning L, Laman M, Mare T, Hwaiwhanje I, Siba P, Davis TME. Accuracy of
14 cerebrospinal leucocyte count, protein and culture for the diagnosis of acute
15 bacterial meningitis: a comparative study using Bayesian latent class
16 analysis. Trop Med Int Health 2014;19:1520–4.

17 Mercado KP, Helguera M, Hocking DC, Dalecki D. Estimating cell concentration
18 in three-dimensional engineered tissues using high frequency quantitative
19 ultrasound. Ann Biomed Eng 2014;42:1292–1304.

20 Ngoyi DM, Menten J, Pyana PP, Büscher P, Lejon V. Stage determination in
21 sleeping sickness: Comparison of two cell counting and two parasite
22 detection techniques. Trop Med Int Heal 2013;18:778–782.

23 O'Donnell M, Miller JG. Quantitative Broadband Ultrasonic Backscatter: an
24 Approach To Nondestructive Evaluation in Acoustically Inhomogeneous
25 Materials. J Appl Phys 1981;52:1056–1065.

26 Ongena K, Das C, Smith JL, Gil S, Johnston G. Determining Cell Number During
27 Cell Culture using the Scepter Cell Counter. J Vis Exp 2010;1–5.

28 Tunis AS, Baddour RE, Czarnota GJ, Giles A, Worthington a. E, Sherar MD,
29 Kolios MC. Using high frequency ultrasound envelope statistics to determine
30 scatterer number density in dilute cell solutions. Proc - IEEE Ultrason Symp
31 2005;2:878–881.

32 Vlad RM, Alajez NM, Giles A, Kolios MC, Czarnota GJ. Quantitative ultrasound
33 characterization of cancer radiotherapy effects in vitro. Int J Radiat Oncol
34 Biol Physiscs 2008;72:1236–1243.

35 Weichselbaum T. An accurate and rapid method for the determination of proteins
36 in small amounts of blood serum and plasma. Am J Clin Pathol 1946;16:40–
37 9.

38 Woessner, S., Lafuente R, Florensa L. La citología óptica en el diagnóstico
39 hematológico. 5th ed. Medici, 2006.

40 Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann a. Automated
41 vs. manual cerebrospinal fluid cell counts: A work and cost analysis
42 comparing the Sysmex XE-5000 and the Fuchs-Rosenthal manual counting

1 chamber. Int J Lab Hematol 2011;33:629–637.

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3

1 **FIGURE CAPTIONS LIST**

2 **Figure 1.** Experimental setup for ultrasound measurements of cell suspensions
3 in ultrafiltrate blood serum as a surrogate of cerebrospinal fluid (CSF).
4

5 **Figure 2.** White blood cell (WBC) concentration measured in a Fuchs-Rosenthal
6 chamber in relation to (left axis) integrated backscatter coefficient (BSC) and to
7 (right axis) BSC-based WBC count. The dashed line represents the linear fit
8 (95%) of WBC concentration to integrated BSC, as provided by the coefficient of
9 determination R^2 . 24 samples were used in total. Two samples with 3 WBC/ μ L
10 and two samples with 7 WBC/ μ L produced almost identical BSC and show as
11 overlapping circles in the plot.
12

13 **Figure 3.** Bland-Altman plot of white blood cell (WBC) concentration measured in
14 a Fuchs-Rosenthal chamber (H) and with the integrated backscatter coefficient
15 (BSC) method (E_{WBC}). The bias (centre-solid line) was of 0 WBC/ μ L and the
16 95% limits of agreement (outer-dashed lines) were -8.11 and 8.11 WBC/ μ L.
17

18 **Figure 4.** Bland-Altman plot of white blood cell (WBC) concentration measured in
19 a Fuchs-Rosenthal chamber (H) and with the integrated backscatter coefficient
20 (BSC) method (E_{WBC}) within concentration levels for meningitis diagnosis.

Table 1. Backscatter power (BSC) in relation to leukocytes (WBC) and erythrocyte concentration for each sample.

BSC (a.u.)	52	55	62	60	60	62	66	66	67	66	69	70	75	72	71	72	82	81	83	84	93	100	107
WBC (#/ μ L)	0	0	2	3	3	5	7	7	10	11	14	17	17	19	20	20	22	23	28	34	53	55	61
ERYTHROCYTES (#/ μ L)	0	0	1	1	1	2	2	3	6	5	2	6	6	7	25	6	8	6	13	8	13	21	13

Figure1

Figure2

Figure3

Figure4