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1	Short-form paper:
2	Flow cytometry to assess CSF fungal burden in cryptococcal meningitis
3	(Running title: Flow cytometry counting of cryptococci)
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5	James E Scriven <sup>1,2,3,4</sup> , Lisa M Graham <sup>3</sup> , Charlotte Schutz <sup>3,4</sup> , Thomas J Scriba <sup>5</sup> , Robert J
6	Wilkinson <sup>3,4,6,7</sup> , David R Boulware <sup>8</sup> , Graeme Meintjes <sup>3,4,6</sup> , David G Lalloo <sup>1,2</sup> , Britta C Urban <sup>1</sup>
7	
8	1. Liverpool School of Tropical Medicine, L3 5QA, UK
9	2. Wellcome Trust Liverpool Glasgow Centre for Global Health Research, L69 3GF,
10	UK
11	3. Clinical Infectious Diseases Research Initiative, Institute of Infectious Disease and
12	Molecular Medicine, University of Cape Town, Cape Town 7925, South Africa
13	4. Department of Medicine, University of Cape Town and Groote Schuur Hospital, Cape
14	Town 7925, South Africa
15	5. South African TB Vaccine Initiative, Institute of Infectious Disease and Molecular
16	Medicine and Department of Paediatrics and Child Health, University of Cape Town
17	7925, South Africa
18	6. Department of Medicine, Imperial College, London, W2 1PG, UK
19	7. Francis Crick Institute, Mill Hill Laboratory, London, NW7 1AA, UK
20	8. Department of Medicine, University of Minnesota, MN 55455, USA
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24	Corresponding author:
25	James Scriven: jscriven@liv.ac.uk
26	(Tel: +44 7876 742428; Fax: +44 151 794 6663)
27	Wellcome Trust Liverpool Glasgow Centre for Global Health Research
28	Block E Royal Infirmary Complex, 70 Pembroke Place, Liverpool, L69 3GF, UK
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# 33 Abstract

Fungal burden in the cerebrospinal fluid is an important determinant of mortality in cryptococcal meningitis but its use to aid clinical decision-making is hampered by the time involved to perform quantitative cultures. Here we demonstrate the potential of flow cytometry as a novel and rapid technique to address this.

## 38 Text

39 Cryptococcal meningitis (CM) remains one of the commonest causes of meningitis in sub-40 Saharan Africa and a significant cause of death among persons with HIV-1 infection (1, 2). Cerebrospinal fluid (CSF) fungal burden is an important determinant of mortality but requires 41 42 quantitative culture – a time consuming process taking several days limiting its usefulness as 43 a clinical decision aid (3). Recent in vitro work using broth dilutions of Cryptococcus 44 neoformans has demonstrated a very close association between the number of cryptococci 45 counted using a flow cytometer and quantitative culture (4). This raises the possibility that 46 flow cytometry might be a useful technique to rapidly assess fungal burden in patients with 47 cryptococcal meningitis. However, no studies have examined this technique on ex vivo 48 samples. We addressed this by performing flow cytometry counting of cryptococci in the 49 CSF of patients with HIV-1-associated CM and compared these counts with measurement of 50 fungal burden using quantitative CSF culture. This study formed part of a larger body of 51 work primarily aimed at examining the CSF immune response in CM.

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53 A prospective cohort study was conducted in Cape Town, South Africa. All participants 54 provided written informed consent; surrogate consent was obtained from the next of kin for 55 patients with impaired consciousness. Ethical approval was obtained from the University of Cape Town and Liverpool School of Tropical Medicine Research Ethics Committees. HIV-56 57 infected persons aged  $\geq 18$  years with a first episode of cryptococcal meningitis (diagnosed by antigen test or culture) were enrolled within 48 hours of diagnosis and lumbar puncture 58 59 performed to measure CSF opening pressure. CSF fungal burden was assessed with 60 quantitative culture (5) and cryptococcal antigen titre (CrAg® LFA, Immy, USA) (6) as 61 previously described. The volume of remaining CSF was measured and the cells pelleted 62 using centrifugation, this was incubated on ice with an amine viability dye (AQUA,

Journal of Clinica Microbiology

ournal of Clinical Microbiology 63 Invitrogen) and anti-CD45-PECy5.5 (Biolegend), then at room temperature with FACS 64 lysing solution (BD Bioscience), protected from light at all times. Cells were fixed using 2% 65 paraformaldehyde and analyzed within 24 hours on a BD LSR Fortessa Flow Cytometer using FACS Diva software (BD Biosciences). A forward scatter threshold of 5,000 was used 66 67 to avoid including any debris in counting; the sample was acquired in its entirety to allow 68 calculation of cell counts. Compensation was performed using species appropriate 69 compensation beads (BD Biosciences; Invitrogen). Data was analysed using Flow Jo version 70 9.5.3 (Tree Star software) [Figure 1]. Cryptococci were defined as CSF cells negative for the 71 pan-leukocyte marker CD45. Counts for the whole sample were divided by CSF volume to 72 obtain Cryptococcus counts per mL CSF. Statistical analyses were performed using Stata 73 version 12 (Stata Corp).

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Sixty HIV-infected patients with cryptococcal meningitis were enrolled with a median CD4
count of 34 cells/µL. CSF samples were available for 58 participants, 36 of whom had not
received any amphotericin B prior to enrolment. The median CSF volume collected for flow
cytometry was 7 mL (interquartile range (IQR), 4.5-8). Median fungal burden was 4.74 log<sub>10</sub>
CFU/mL (IQR 3.5-5.75) measured by quantitative culture and 4.53 log<sub>10</sub> *Cryptococcus* yeasts
per mL (IQR 3.33-5.21) measured by flow cytometry. Median CrAg® LFA titre was 1:8000
(IQR 2000-16000).

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Cryptococcal counts measured by flow cytometry were strongly correlated with both quantitative culture (Pearson's r=0.91, p<0.0001) [Figure 2a] and CrAg titre (Spearman rho=0.75, p<0.0001). Linear regression showed that quantitative culture result could accurately be predicted from flow cytometry counting ( $log_{10}$ CFU/ml = 1.31 x  $log_{10}$  flow count – 1.28; R<sup>2</sup>=0.82, p<0.0001). The agreement between flow cytometry counting and 88 quantitative culture was also assessed using a Bland-Altman plot [Figure 2b]. This showed 89 good agreement between these two measurements with a mean difference of  $-0.1 \log_{10}$ 90 CFU/mL and only 6.9% (4/58) of values outside the 95% limits of agreement. These outlying values were mainly among participants with low fungal burdens (<500 CFU/mL) where flow 91 92 cytometry counting produced values that were approximately 1-2 log<sub>10</sub> CFU/mL higher. A 93 similarly strong correlation and agreement between the two measurement techniques was also 94 noted when analysis was restricted to the 36 participants who had not received any anti-95 fungal therapy prior to CSF sampling (Pearson's r=0.93, p<0.0001 [Figure 2c]; mean difference -0.30 log<sub>10</sub> CFU/mL, with 11.1% (4/36) of values outside the limits of agreement) 96 97 [Figure 2d].

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99 These findings suggest that flow cytometry has the ability to provide a rapid and accurate 100 measurement of fungal burden in persons with HIV-associated cryptococcal meningitis. If 101 combined with a cryptococcal viability stain (as previously demonstrated *in vitro* (4)) flow 102 cytometry could also be used to assess the response to treatment.

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104 Due to the well-recognized toxicity of amphotericin B (7), there is considerable interest in 105 short course regimens particularly in those patients with low fungal burden (8). Results from 106 quantitative culture are not available in a timely enough manner to inform clinical decision 107 making, but the rapidity of the result obtained from flow cytometric cryptococcal counting 108 could potentially overcome this problem. This could allow for the reduction of both drug 109 toxicity, cost and duration of hospitalization. Although this technique does require access to a 110 flow cytometer, suitable machines are available in many laboratories in sub-Saharan Africa 111 where they are used to measure CD4 count. In areas where they are not available, rapid 112 assessment of fungal burden might be possible using quantitative microscopy (4).

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There were a number of limitations to this study. No cryptococcal specific stain was used to 114 identify cryptococci, instead they were assumed to be any CD45 negative cell found in the 115 116 CSF. Given that all participants had laboratory confirmed cryptococcal meningitis, a lysis 117 buffer was used to ensure no erythrocytes remained in the CSF, and host leukocytes were excluded using the pan-leukocyte marker CD45, we feel this is a reasonable assumption and 118 119 that our results are valid. However, to be a fully robust clinical assay an anti-cryptococcal 120 stain would ideally be incorporated into the panel and the assay validated on CSF samples 121 from patients who do not have CM. This would have the additional benefit of improving the 122 accuracy of flow cytometric counting at low fungal burdens where debris or miscellaneous 123 cells may have interfered with the counting process. In addition, we only examined the use of 124 flow cytometric counting to assess fungal burden at baseline and did not assess changes on 125 anti-fungal therapy. Future work should aim to incorporate a cryptococcal viability marker to 126 address this issue as previously assessed in vitro (4).

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### 148 **Conflict of Interest statement**

149 None of the authors have any conflict of interest to declare.

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Figure 1. CSF flow cytometry gating (a1) CSF cells (FSC-SCC) - poor definition of cell 155 subsets due to cryptococci; (a2) CD45 and live-dead stain are used to separate cells; (b) 156 FSC-SCC plot of cryptococci; (c) FSC-SCC plot CSF leukocytes; (d) FSC-SCC plot dead 157 158 CD45+ cells.

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163 Figure 2. (a) Scatterplot demonstrating association between flow cytometry counting and 164 quantitative culture; (b) Bland-Altman plot showing good agreement between flow counting 165 and quantitative culture (greyed area indicates the 95% limits of agreement); (c) Association 166 between flow counting and quantitative culture among participants who had not received antifungal therapy before CSF sampling; (d) Bland-Altman plot showing agreement between 167 168 the two techniques limited to participants who had not received antifungal therapy before 169 CSF sampling (greyed area indicates the 95% limits of agreement).

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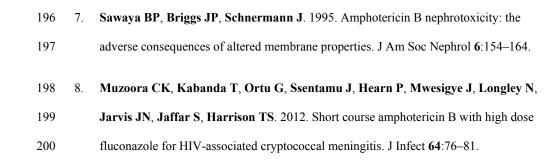
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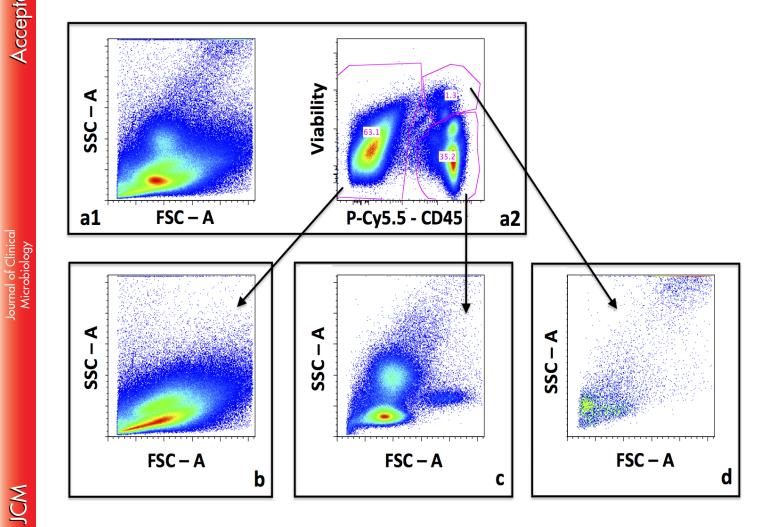
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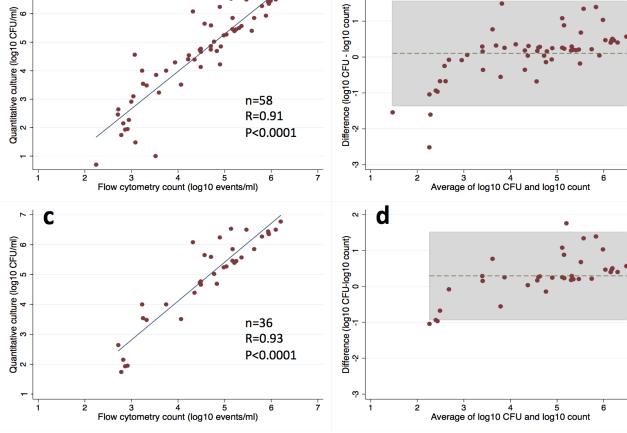
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