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A Novel Flow Cytometric Protocol for Assessment of Yeast Cell Adhesion

A. Silva-Dias,[1,2] * I. M. Miranda,[1,2] R. Rocha,[1] M. Monteiro-Soares,[3] A. Salvador,[4] A. G. Rodrigues,[1,2,5] C. Pina-Vaz[1,2,6]

[1]Department of Microbiology, Faculty of Medicine, University of Porto, Portugal

[2]Cardiovascular Research and Development Unit, Faculty of Medicine, University of Porto, Portugal

[3]Department of Biostatistics and Medical Informatics, Faculty of Medicine, CINTESIS, University of Porto, Portugal

[4]ESTeSL—Higher School of Health Technology of Lisbon, Polytechnic Institute of Lisbon, Portugal

[5]Burn Unit and Department of Plastic and Reconstructive Surgery, Hospital S. João, Porto, Portugal

[6]Department of Microbiology, Hospital S. João, Porto, Portugal

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*Correspondence to: A Silva-Dias, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

Email: asilvadias@med.up.pt

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Abstract

Microbial adhesion is a field of recognized relevance and, as such, an impressive array of tools has been developed to understand its molecular mechanisms and ultimately for its quantification. Some of the major limitations found within these methodologies concern the incubation time, the small number of cells analyzed, and the operator's subjectivity. To overcome these aspects, we have developed a quantitative method to measure yeast cells' adhesion through flow cytometry. In this methodology, a suspension of yeast cells is mixed with green fluorescent polystyrene microspheres (uncoated or coated with host proteins). Within 2 h, an adhesion profile is obtained based on two parameters: percentage and cells-microsphere population's distribution pattern. This flow cytometry protocol represents a useful tool to quantify yeast adhesion to different substrata in a large scale, providing manifold data in a speedy and informative manner. © 2011 International Society for Advancement of Cytometry

Key terms

adhesion; yeast; cytometry; quantitative methodology

IN nature, microorganisms such as bacteria and fungi prefer a community-based and sedentary lifestyle, usually binding to biotic or abiotic surfaces. This ubiquitous behavior confers obvious advantages for microbial development and proliferation, such as the possibility to establish symbiotic relationships or the development of complex structures like biofilms. Biofilm formation constitutes a protective milieu against environmental injuries or human host defenses (1–5). From an evolutionary perspective, adhesion ability has been considered a selective advantage providing microorganisms the chance to emerge as surface-bound populations, through cell–cell interactions or bindings (6).

Surface adhesion is a complex and multiphase process, dictated by a number of variables including organism species, surface composition, and environmental factors. Typically, it starts by a primary and reversible phase, which is highly dependent on physiochemical interactions, namely hydrophobic ones. Subsequently, it evolves to a second irreversible phase mediated by specific adhesins, with specific molecular interactions occurring between the organism and the surface (6–9). Both interactions are strongly biased by the cell wall proteome of each microorganism.

Yeast cell wall possesses hydrophobic proteins embedded in their matrix, which mediate the initial fungal attachment. Hydrophobic cells tend to bind in a great number to plastics, host proteins (laminin, fibrinogen, and fibronectin (FN)), and host tissues than the hydrophilic ones (7). Another important class of specialized cell wall proteins involved in adhesion is the glycosylphosphatidylinositol proteins referred to as "adhesins." Despite sharing the same structure, an N- and a C-terminal interspersed by a tandem repeat region, fungal adhesins, differ in their capacity to adhere to a specific surface. According to the different hosts and environmental conditions, adhesion genes can be expressed differentially (9–11).

The efficiency of microbial adhesion has huge impact at various levels, namely ecological, industrial, and clinical, with either positive or negative repercussions. Bioremediation, nitrogen fixation, flocculation of industrial brewing, and wine production are just a few examples of how microbial adhesion can enhance the performance of an industrial process (10–12). Clinically however, microbial adherence poses serious concerns and can lead to critical consequences. Pathogenic bacteria and fungal cells can adhere to host tissues or to biomaterial used in common medical indwelling devices leading to biofilm formation, which frequently results in bloodstream infections associated to high mortality rates (13–15). In fact, adhesion is considered a significant virulence attribute as it is determinant for colonization, invasion, and establishment of disease.

Extensive research has been performed to dissect the molecular mechanisms underlying microbial adhesion, and several methods have been developed to quantify adhesion. Overall, these methodologies are laborious, expensive, and require sophisticated equipment not always available in laboratories. Nonetheless, the major drawback associated to the assays so far described, concerns the subjectivity of a quantification performed by the investigator. Moreover, output data frequently corresponds to a global measurement of adhesion ability displayed by a microbial population without taking into account the variability existing within the population.

To overcome some of the limitations described, we aimed to develop a novel quantitative method to measure cell-surface adhesion through flow cytometry. Essentially, the novel methodology here described is an adaptation of the protocol described by Hazen and LeMelle (16) to quantify hydrophobicity using microscopy. Briefly, yeast cells are allowed to interact with microspheres and the relative population hydrophobicity is calculated as a percentage of yeast cells with three or more attached polystyrene microspheres. We replaced the operator microscopic counting by flow cytometry. The adhesion assay described herein is based on a simple principle: yeast cells became fluorescent when attached to highly green fluorescent microspheres. Therefore, by flow cytometry, a quantitative distinction between nonadherent yeast cells (nonfluorescent) and adherent cells (fluorescent) is achieved.

Cytometry is extensively used to characterize eukaryotic cells. Several microbiologic applications have been described, namely in detection, evaluation of viability, and susceptibility profile of microorganisms (17–21). Although it proved to be beneficial over conventional methods, the potential of this tool is still underestimated. Recently, flow cytometric adherence assays have been used to measure the interaction between bacteria and fungi with eukaryotic cells (22–24).

Herein, we optimized a novel flow cytometry protocol to measure yeast cell adhesion ability toward abiotic and biotic surfaces in a quantitative, fast, and informative manner.

Material and Methods

Strains and Cultures

Candida albicans SC5314, *Candida parapsilosis* OL021 (clinical isolate), *Saccharomyces cerevisiae* S150-2B; four *S. cerevisiae* strains expressing *C. albicans* adhesins, Als3p, Als5p, Als6p, and Als7p, and one *S. cerevisiae* carrying the empty plasmid (pADH) were used in this study (11). *S. cerevisiae* S150-2B and plasmids carrying Als proteins were kindly offered by Dr. John E. Edwards Jr. and Prof. Scott G. Filler. As its adhesion profile is well characterized, these strains were used to validate this novel methodology.

All strains were kept frozen in YPD with 40% glycerol at −70°C. For all assays, yeast cells were grown overnight with agitation in YPD broth at 30°C. *S. cerevisiae* expressing *C. albicans* adhesins were grown in minimal medium (1x yeast nitrogen base broth, 2% glucose, and 0.5% ammonium sulfate, supplemented with 100 μg/ml L-leucine, L-tryptophan, L-histidine, and adenine sulfate).

Optimization of Yeast Flow Cytometric Microsphere Adhesion Assay

After growing overnight at 30°C, yeast cells were harvested and washed twice with phosphate buffer saline (PBS) 0.01 M (NaCl 0.138 M; KCl −0.0027 M; pH 7.4; Sigma). A suspension of 2×10^6 yeast ml^{-1} was prepared and mixed with carboxylated highly green fluorescent polystyrene microspheres (1 μm; F-8823; Molecular Probes) at final concentration of 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 microspheres ml^{-1}. The mix of microspheres and yeast was incubated at room temperature for 15, 30, 90, and 120 min, with agitation (150 rpm). Single yeast cell suspensions and microspheres suspensions were used as controls. Following incubation, each suspension was vortexed and 50,000 events were analyzed by flow cytometry in propylene tubes. To validate cytometric results, epifluorescence microscopic examination of each sample was performed in parallel to flow cytometric analysis. Yeast cells were stained with calcofluor (0.05% vol/vol, Fluka), and images were taken with a fluorescence microscope Axioplan Zeiss, coupled with acquisition image system AxioVision (Zeiss) using two different filters (DAPI: excitation $\lambda = 365$ nm; emission $\lambda = 445/50$ nm and Alexa 568: excitation $\lambda = 530/585$ nm; emission $\lambda = 615$ nm).

Flow Cytometry Analysis

A standard flow cytometer (FACSCalibur, BD Biosciences, Sydney) with three PMTs equipped with standard filters (FL1: BP 530/30 nm; FL2: BP 585/42 nm; FL3: LP 670 nm), a 15 mW 488 nm Argon Laser and operating with cell Quest Pro software (version 4.0.2, BD Biosciences, Sydney) was used. The Flow Cytometry data file format used was FCS 2.0[a].

Acquisition settings were defined using carboxylated highly green fluorescent polystyrene microsphere samples by adjusting voltage to the third logarithmic (log) decade of all fluorescence channels (Fig. 1a). FSC was used as trigger signal. Samples were analyzed in the FL3 fluorescence channel (fluorescence channel where all analyzed populations are clearly distinct), using two dot plots: SSC versus FSC and SSC versus FL3 (Fig. 1). Results were expressed using two parameters: (a) percentage of cells with microspheres attached and (b) distribution pattern.

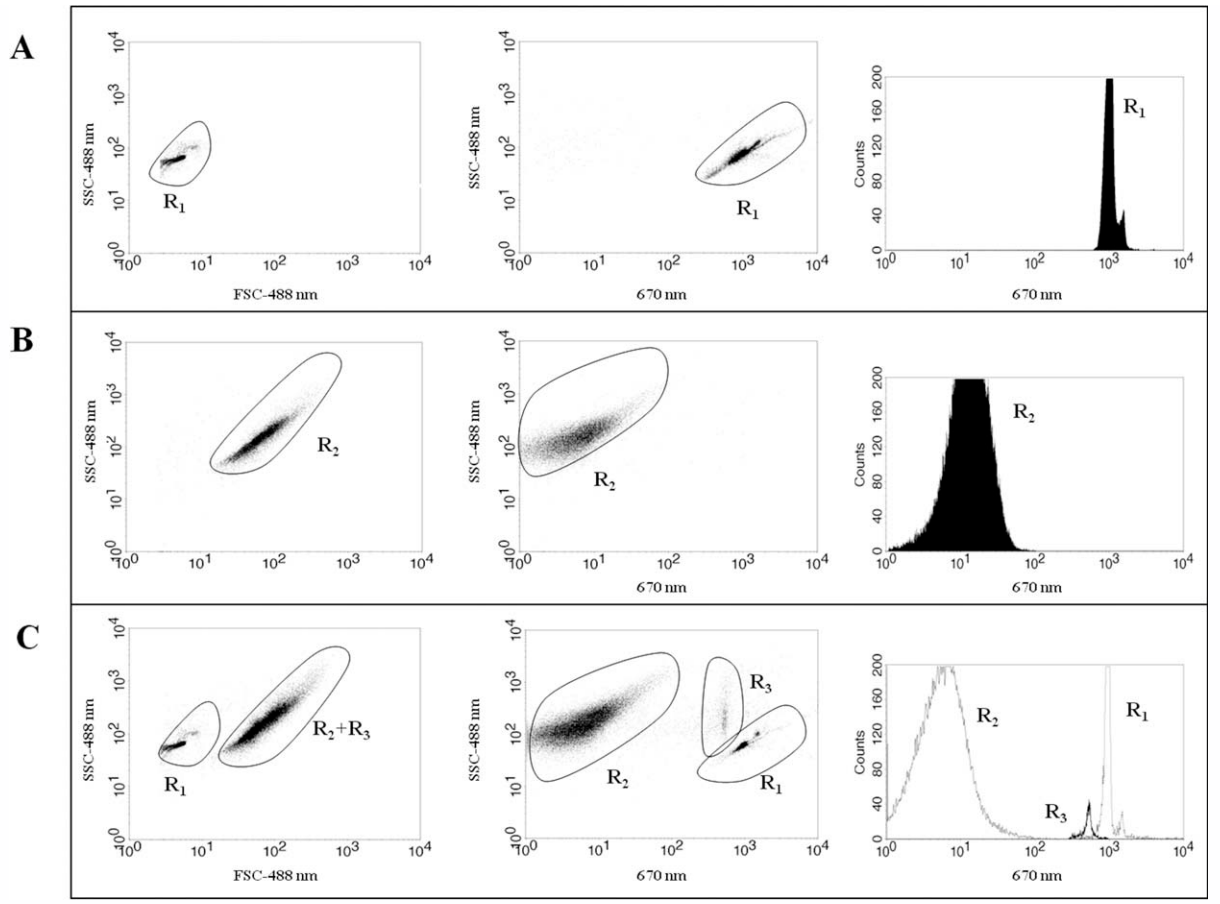

Figure 1. Flow cytometry analysis. (**A**) Representation of single microspheres population (R1) adjusted to the third logarithmic decade. (**B**) Discrimination of yeast cell population (R2). (**C**) Analysis of a sample containing yeast cells (1×10^6 cell ml^{-1}) plus microspheres (1×10^7 microspheres ml^{-1}) after 30 min incubation. Differentiation of R2 and R3 populations was achieved in a dot plot of SSC versus FL3.

Coating of Fluorescent Polystyrene Latex Microspheres

FN and gelatin (GEL) (Sigma-Aldrich) were covalently coupled to microspheres using the carbodiimide method as recommended by microsphere manufacturer (Molecular Probes). Briefly, the FN or GEL solutions were prepared in MES buffer at the required concentration (0.01 mg ml^{-1}) and 5 ml of microsphere stock solution (2% aqueous suspension of microspheres) was added. On 15 min of room temperature incubation, EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) (4 mg ml^{-1}) was mixed with the coating microspheres solution. To quench the reaction, glycine was added to a final concentration of 100 mM, and incubated for 2 h in an orbital shaker. Samples were centrifuged (5000*g*, 20 min), washed three times with PBS and resuspended in PBS with 1% bovine serum albumin (BSA).

Statistical Analysis

Distribution normality was assessed by evaluating the histogram and applying the Kolmogorov–Smirnov (KS) nonparametric test. All variables presented a normal distribution and a $P > 0.05$ in the KS test. Subsequently, we have used the Student's *t* test (a two-tailed analysis) for the Mean comparison between *S. cerevisiae* pADH and *S. cerevisiae* expressing *ALS* genes.

For all analyses, significance was defined as $P < 0.05$. All statistical analyses were performed using the SPSS version 17.0 (SPSS, Chicago, IL).

This manuscript follows the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) standard. A detailed description of the experimental design, methodologies, results and conclusions is included in the supporting information.

Results

Yeast Flow Cytometric Microsphere Adhesion Assay

Yeast and microspheres discrimination. Microspheres attachment does not cause a substantial change in either size or complexity of yeast, and as such, the subpopulation of yeast cells adherent to microspheres is not obvious (Fig. 1c). Three subpopulations can be distinctly identified in the dot plot

mode of SSC versus fluorescence: R1-microspheres, R2-yeast cells, and R3-yeast cells adherent to microspheres (Fig. 1c).

Analysis was focused on the yeast-microsphere subpopulation (R3) and so, only the *Candida* population was gated on the dot plot SSC versus FSC, excluding the microspheres population (R1).

Microsphere concentrations. To optimize microsphere concentration for adhesion test preventing limitated amount of microsphere (which may lead to a reduced yeast adhesion profile), several concentrations ranging from 1×10^5 to 1×10^9 microspheres ml^{-1} were tested. Concentrations below 1×10^7 microspheres ml^{-1} were minimal compared with the concentration of yeast cells (1×10^6 cells ml^{-1}); this unbalanced yeasts/microspheres relation caused a negative bias in the quantification of adhesion. We found a direct relation between yeast adhesion and microsphere concentration until reaching a saturation point with the concentration 1×10^7 microspheres ml^{-1} (Fig. 2). Concentrations above 1×10^7 microspheres ml^{-1} showed no differences in the percentage or in the distribution pattern of R3 population; therefore, this concentration was selected for further procedures. Incubation time was set to 30 min, allowing adhesion without microsphere or yeast aggregation (Fig. 2).

Adhesion profile. Adhesion profiles were determined based on a multifactorial analysis of the R3 subpopulation according to two parameters (Fig. 3): (i) percentage of gated population, representing the number of yeast cells with microspheres attached and (ii) distribution pattern of a population, classified as either homogenic or heterogenic. A homogenous distribution pattern indicates a population, wherein yeast cells are bound to the same number of microspheres (frequently binding to a single microsphere) (Fig. 3). This population peak is approximately superimposed to that of the microspheres population (Fig. 1c). In contrast, a heterogeneous pattern displays the presence of different peaks beyond the third logarithmic decade and means attachment of more than a single microsphere to each cell (Fig. 3).

To establish these patterns, a *S. cerevisiae* S150-2B (11) strain displaying low adhesion profile (1.62% of adhered cells and a homogenic distribution pattern) was used as negative control. As a positive control, we selected a *C. parapsilosis* clinical strain, isolated from a central venous catheter, displaying a high adhesion profile (35% of adhered cells and a heterogenic distribution pattern, with 44.39% of the yeast population found beyond the third logarithmic decade).

Adhesion of *S. cerevisiae* Expressing Als Proteins

This flow cytometry adhesion protocol was validated with *S. cerevisiae* strains previously characterized for their adhesion phenotypes through standard methodology (11). Thus, the adhesion ability of *S. cerevisiae* expressing *C. albicans ALS* adhesion genes was assessed toward FN and GEL. *ALS* (agglutinin-like sequence) genes encode a family of adhesins involved in multiple host-pathogen interactions (attachment

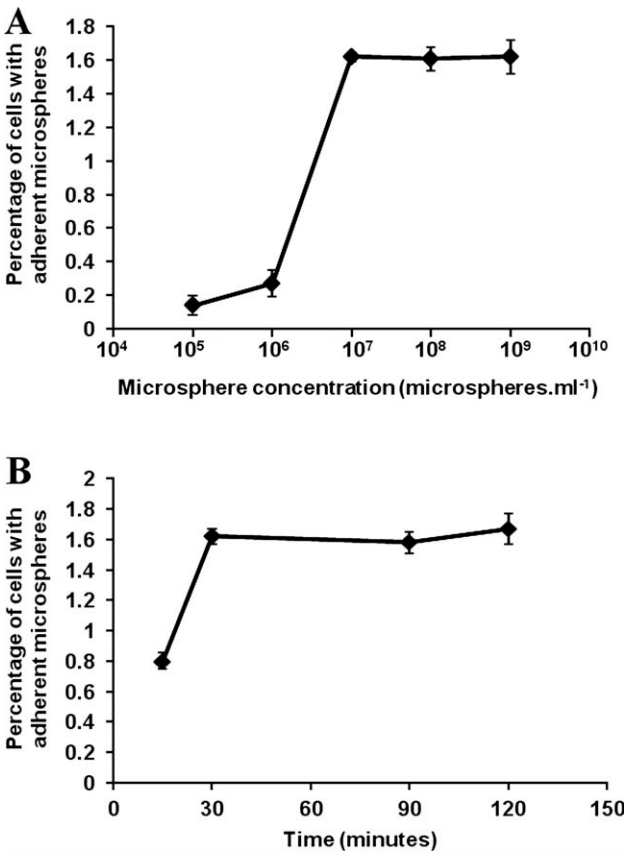

Figure 2. Kinetics of cell-microsphere attachment. (**A**) For optimization of microspheres concentration, 1×10^6 yeast cell ml^{-1} were incubated with 1×10^5 to 1×10^9 microspheres ml^{-1} for 30 min. Concentrations below 1×10^7 microspheres ml^{-1} proved to be insufficient for adhesion quantification. Above this concentration, no differences were found in the percentage of cells with adherent microspheres or in the distribution pattern (data not shown). (**B**) To determine incubation time, 1×10^6 cell ml^{-1} were incubated with 1×10^7 microspheres ml^{-1} for periods of time ranging from 15 to 120 min. Thirty minutes of incubation was the selected time.

to epithelial cells, endothelial cells, and extracellular matrix proteins), also playing an important role in biofilm formation and in the mediation of other cellular processes like iron acquisition (25,26). As described by Sheppard et al. (11), *S. cerevisiae* clones expressing *ALS3*, *ALS5*, *ALS6*, and *ALS7* genes analyzed by flow cytometry revealed distinct adhesion profiles, which also varied according to the host proteins (Table 1). The negative control, *S. cerevisiae* transformed with pADH (empty plasmid), exhibited low adhesion profile for both host proteins (11).

Als3p and Als5p strains displayed the highest adhesion profile. Als3p yielded the higher percentage of cells with adherent microspheres as well as the higher relative number of microspheres *per* cell (Table 1). Although a higher percentage of Als3p cells adhered to FN, a similar distribution pattern was obtained for FN and GEL. No differences in adhesion to both proteins were detected for Als5p cells.

The Als6p strain displayed differential adhesion profiles for GEL and FN. The percentage of cells adherent to micro-

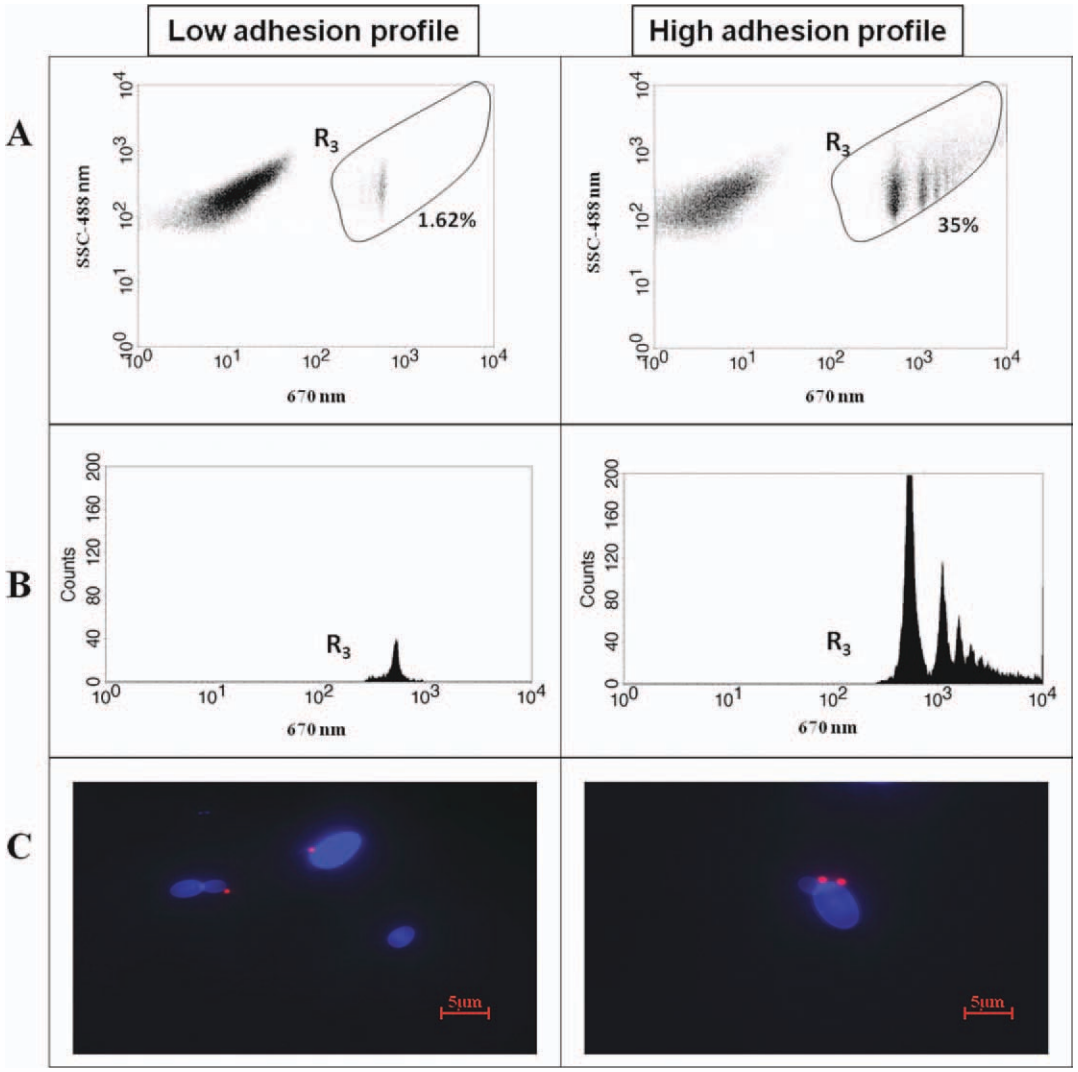

Figure 3. Characterization of adhesion profiles. A multifactorial adhesion profile can be defined for each sample based on two parameters of the R3 population: percentage of cells with adherent microspheres and distribution pattern. The left panel represents *S. cerevisiae* S150-2B strain exhibiting a low adhesion profile used as a negative control; the right panel depicts a representative case of a high adhesion pattern, displayed by a *C. parapsilosis* clinical isolate. (**A**) shows the percentage of cells with adhered microspheres for the different profiles; (**B**) represents the characteristic distribution pattern (homogenic versus heterogenic) for a low adhesion profile (left panel) and a high adhesion profile (right panel). Representative images of cells with adhered microspheres belonging to populations displaying the different adhesion profiles are included in (**C**).

Table 1. Adhesion ability displayed by *S. cerevisiae* pADH and *S. cerevisiae* expressing *C. albicans ALS* genes toward host proteins: gelatin (GEL) and fibronectin (FN)

	PERCENTAGE OF CELLS WITH ADHERENT MICROSPHERES		DISTRIBUTION PATTERN	
	GEL	FN	GEL	FN
pADH	0.96 ± 0.21	1.6 ± 0.27	Homogenic	Homogenic
Als3p	5.4 ± 0.88*	10.3 ± 2.22*	Heterogenic	Heterogenic
Als5p	6.6 ± 1.20*	8.0 ± 1.03*	Heterogenic	Heterogenic
Als6p	4.8 ± 0.61*	1.7 ± 0.10	Heterogenic	Homogenic
Als7p	1.6 ± 0.15*	2.4 ± 0.23*	Homogenic	Homogenic

Adhesion was quantified through analysis of two parameters: percentage of cells with adherent microspheres and distribution pattern. Data represents the mean of at least three experiences performed in triplicate.

* $P < 0.05$ ALS when compared with pADH.

spheres coated with GEL was four-fold higher, when compared with control, and a heterogeneous pattern was obtained. Conversely, the adhesion ability of Als6p cells to attach to FN microspheres was similar to that of pADH, providing evidence that *C. albicans* Als6p is not involved in FN binding (Table 1).

Als7p adhesion profile was similar to that of pADH both for GEL and FN.

Discussion

The adhesion assay described in our study is based on a simple principle: yeast cells become fluorescent when attached to fluorescent microspheres. By flow cytometry, we were able to distinguish nonadherent (nonfluorescent) from adherent yeast cells (fluorescent). In this protocol, microspheres act as a surface to which yeast cells can adhere enabling their quantification.

Compared with classical methodologies, flow cytometry adhesion measurements exhibit major improvements: (i) large scale quantitative analysis with possible detection of residual adhesion; (ii) automated analysis which prevents operator's subjectivity and avoids false positives, as yeast cells with truly adherent microspheres are evaluated as one single event; (iii) results within a short period of time, in contrast to classical methodologies that usually take several hours; (iv) finally, a multifactorial analysis based in features displayed by individual cells is provided allowing further evaluation of the adhesion profile of the adherent population (heterogeneous/homogenous). Thus, concomitant analysis of two independent parameters by cytometric analysis (percentage of cells with adherent microspheres and distribution pattern) enables an extensive characterization of each sample population. Furthermore, this assay constitutes a useful and versatile tool to characterize yeast attachment to a wide variety of substrata or molecules of interest (by coating the microsphere). While optimizing this methodology, polystyrene microspheres were used (a representative of plastic). These microspheres can be easily coated with a wide range of molecules, therefore allowing to measure adhesion to other components, like host constituents.

After coating the microspheres with FN and GEL and using flow cytometry protocol, the adhesion profile of *S. cerevisiae* expressing *C. albicans* adhesins (*ALS3*, *ALS5*, *ALS6*, and *ALS7* genes) was determined. Als proteins are a major group of adhesins encoded by the *ALS* gene family. It is known that these cell surface proteins mediate adhesion to different host molecules (namely FN, GEL, laminin, and cellular lines), yeast aggregation and potentiates biofilm formation (11,25,26). This approach allowed us not only to demonstrate the easiness of the bead coating process but also to compare the adhesion profile obtained by flow cytometry with the one previously obtained by Sheppard et al. (11) using the six-well plate assay method. Regarding their adhesion profile, these strains were already well characterized. Data obtained by Sheppard et al., 2004 (11), with a laborious classical methodology that takes 48 hours of incubation, were compared to the percentage of cells with adherent microspheres determined by flow cytometry and results were very similar.

In conclusion, our adhesion flow cytometric method proves to be a step forward regarding adhesion methodologies, providing broader information within shorter time periods, with considerably less laboratorial manipulation. Furthermore, it allows the characterization of each cell population based on its adhesion profile, which proves to be important in clinical and environmental fields.

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