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# Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores

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ABSTRACT

Flow cytometry is often used for viability and vitality assessment in bacteria and yeasts. However, its application to the study of fungal spore development is uncommon, probably due to the difficulties in successfully staining these cells.

In the current study, we used flow cytometry for the first time to assess the effects of a disinfection treatment on the survival, growth and metabolic activity of fungal spores (Penicillium chrysogenum, Aspergillus nidulans and Aspergillus niger) submitted to gamma radiation (0–15 kGy). The Forward and Side-Scatter parameters of the cytometer were used to assess the differences in size and complexity of particles. Furthermore, two fluorescent dyes were used: Propidium Iodide to assess the membrane integrity and spore viability, in a culture-independent procedure; and Dihydroethidium to measure the changes in metabolic activity of irradiated spores in their first 10 h of growth in a liquid culture medium.

Our results support that flow cytometry is a valuable tool in assessing different biological parameters and biocide effects, as it allowed accurate determination of the viability, growth and metabolic activity of gamma-irradiated spores. The fluorescence of Propidium Iodide was 5–7× more intense in unviable spores. The Dihydroethidium fluorescence increase was associated with faster growth. Control and low radiation doses allowed the germination and growth of spores, while higher doses led to growth inhibition and lower fluorescence.



## 1. Introduction

Flow cytometry (FCM) allows the simultaneous analysis of several characteristics of particles from a heterogeneous population (size, complexity, fluorescence). The light scattered by each particle is filtered, and routed to appropriate detectors that measure the magnitude of pulses, which represent the amount of scattered light. Flow cytometry has many different applications in several fields of research: blood cell counts, viability and vitality of both bacteria and yeasts and microbial discrimination (Davey and Kell, 1996; Veal et al., 2000; Davey, 2002). Each particle is analysed individually, and several hundred can be analysed each second, providing information on single particle properties instead of population averages. It is therefore a powerful analysis tool for qualitative and quantitative purposes.

The size and complexity of particles is assessed using light-scattering measurements; forward-scattered light (FSC) provides information on the particle size, whereas side-scattered light (SSC) is associated with particle complexity. However, using fluorescent stains and probes, many other cell properties, and cell contents can be analysed. According to the flow cytometer settings and stain specifications, various fluorochromes can be used simultaneously to assess different particle parameters (Shapiro, 2003). The use of multiple parameters to detect and select different particles is one of the technical advantages of flow cytometry, as one can differentiate fungal propagules from biotic debris as well as other cells (Prigione et al., 2004). These analyses can be performed right after a given treatment is applied, without the need to re-inoculate, incubate and perform colony counts, in a process that would usually require several days.

Propidium Iodide is a fluorescent dye that binds to DNA, and is generally unable to penetrate the membrane of live cells, however it stains dead cells, working as a dye-exclusion viability probe

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(Williams et al., 1998; Steinkamp et al., 1999) and it is suitable for the study of fungal conidia (Brul et al., 1997). When excited by a 488–523 nm laser, it fluoresces orange-red, and can be detected using a 562–588 nm band pass filter. It is commonly used in the evaluation of cell viability or to assess DNA content in cell cycle analysis by flow cytometry. Experiments with bacterial cells show that PI staining is usually independent of cell growth phase, and that cells can be stored in glycerol for long periods while retaining the stain (Williams et al., 1998).

Dihydroethidium is a chemically reduced ethidium derivative that has no positive charge. When chemically reduced, this dye exhibits blue fluorescence within the cytoplasm. In viable cells, it can be oxidized to ethidium by reactive oxygen species (ROS), which intercalates with DNA, fluorescing red (Breeuwer and Abee, 2000). In general, spores and conidia have thick and resistant cell walls to protect protoplasts from physical, chemical, and biological damage, but sometimes this inhibits effective staining (Prigione et al., 2004). In this study, controls were made using an epifluorescence microscope (Nikon Optiphot) to confirm that the fluorochromes were actually entering the cells when expected.

Gamma rays are electromagnetic waves with high penetrating power that are able to pass through materials without leaving any harmful residue, which is a big advantage when compared with other disinfection treatments (Adamo et al., 1998, 2001; Da Silva et al., 2006). This sterilization treatment directly damages cell DNA through ionization, induces mutations, and ultimately kills the cell. Through the radiolysis of cellular water, it also promotes the formation of reactive oxygen species (ROS), free radicals and peroxides that cause single and double strand DNA breakages (McNamara et al., 2003). Fungi have successfully been inactivated from different materials, such as paper, wood and soil, using radiation doses ranging from 6 to 15 kGy (Hanus, 1985; Pointing et al., 1998; McNamara et al., 2003; Da Silva et al., 2006).

Reactive oxygen species are also formed in the course of metabolic activity, and can therefore be used as metabolic indicators, since aerobic energy transduction depends on a complex electron transport chain and a proton pumping system in the mitochondrial membrane, which is susceptible to electron leakage (Bradner and Nevalainen, 2003). This leads to the formation of the superoxide anion, which is a ROS that can promote the formation of other ROS molecules, and at higher concentrations, they can damage cellular components and lead to cellular dysfunctions (Osiewacz, 2002). They may serve as regulators of fungal population development, for example, by inhibiting growth in excessively dense spore suspensions. In fact, self-suppression of germination in dense spore suspensions can be explained by the deficiency of vital resources, and self-intoxication with vital activity products: germinating spores produce ROS, which can suppress spore development.

Some fungi cope with increasing ROS concentrations using different strategies: by decreasing the surface area; by developing other mechanisms to limit the penetration of external substances. Melanin and other anti-oxidants, eventually help to balance the formation of ROS inside the cell. Reactive oxygen species regulate the most vitally important processes in fungi: phase development change, intercellular communications, and protection from interspecies competition (Gessler et al., 2007).

The aims of this work were to analyse the viability of irradiated fungal spores with flow cytometry without the need to culture them; and, coincidently, to assess the metabolic activity and growth of fungal spores in their first 10 h of growth in a liquid culture medium. Different doses of gamma radiation (0–15 kGy) were applied to fungal spores of three different species – *Penicillium chrysogenum, Aspergillus niger* and *Aspergillus nidulans* – whose strains were isolated from library documents in a previous work (Mesquita et al., 2009). They are common contaminant fungi of documents, and have been reported in different substrates: paper, parchment, leather, textiles and film (Zyska, 1997; Sterflinger, 2010; Sterflinger and Pinzari, 2011). All three species are potentially harmful to humans (Bennett and Kilch, 2003; Samson et al., 2010).

Most microorganisms living in, or on, objects of art usually depend on special nutrients, so only a minority of species can be cultivated under laboratory conditions (Scharbereiter-Gurtner et al., 2001). Cultivation-independent methods enable the assessment of slower growing or even non culturable microorganisms and, in some cases, render the analysis faster and less expensive.

## 2. Materials and methods

### 2.1. Tested fungal species

Three fungal species were selected for this study – *P. chrysogenum*, *A. niger* and *A. nidulans.* They were isolated previously by Mesquita et al. (2009). *P. chrysogenum* is a ubiquitous cellulolytic fungus that is a halotolerant, mesophile and psychrotolerant species. Both *A. niger* and *A. nidulans* are cosmopolitan cellulolytic species that are also xerophilic and mesophilic, although they are able to grow at warm temperatures.

### 2.2. Spore suspensions

The three isolates were re-cultured in PDA medium plates, and incubated at 28 °C until sporulating cultures were obtained (6–8 days). Fresh conidia were harvested by washing with a sterile saline solution (0.9% w/v) containing Silwet L-77 surfactant (0.01% v/v) (GE Silicones, USA), and this fluid was filtered using 10 μm Partec CellTrics filters (Partec, Germany) to remove the residual mycelia. To avoid the presence of particles which were smaller in size, washing of the fungal plates was performed gently to exclude as much debris as possible from the spore suspensions.

The presence of spores was confirmed using an optical microscope. The original concentration of the spore suspensions was estimated using the Neubauer chamber method, and adjusted to $10^7$ spores/ml. Samples were stored in the cold (4 °C) and without light until the irradiation procedure.

### 2.3. Irradiation procedure

Spore suspensions were prepared in triplicate for each species tested, using 2 ml micro tubes, and these were irradiated in a Cobalt 60 experimental source (Precisa 22) located at the Nuclear and Technological Institute campus (Sacavém, Portugal). Five gamma radiation doses were applied: 3, 6, 9, 12 and 15 kGy, using a dose rate ranging between 2.45 kGy/h and 2.87 kGy/h. Absorbed doses were monitored using calibrated routine dosimeters (maximum variation range of ±2.5%; Perspex, Harwell). After the irradiation procedure, the tubes containing the irradiated spore suspensions were stored in the cold (4 °C) and without light until the flow-cytometry analyses.

### 2.4. Flow cytometric analysis

Growth, viability and metabolic activity of fungal spores were assessed in a Partec CyFlow Space (Partec, Germany) flow cytometer, equipped with a green solid-state laser (532 nm/30 mW). The flow rate was adjusted to approximately 300 particles $s^{-1}$. To remove any particles smaller than spores from the analysis, the discriminator was defined for the particle size (FSC) just under the lowest spore signals. The obtained cytograms were analysed using FlowJo 8 software suite (www.flowjo.com), in particular, debris and cell fragments were removed by gating.