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SHORT COMMUNICATION

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A simple method for isolation of nuclei from *Basidiobolus ranarum* (Zygomycota)

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Abstract A simple method for isolating the nuclei from *Basidiobolus ranarum* was established. To improve the yield and purity of nuclei, we investigated maceration methods, buffer composition, and centrifugation conditions to establish an optimal procedure. *Basidiobolus ranarum* cultured for 5 days was enzymatically macerated and then homogenized and filtrated through stainless steel sieves. The crude cell homogenate was loaded on a layer of buffer containing 50% glycerol and centrifuged at 1500 *g*. The resultant pellet contained pure nuclei.

Key words Cell fractionation · Glycerol density-gradient centrifugation · Nucleus

Isolation of nuclei is frequently needed in cell biology. Isolated nuclei are an ideal material for investigations such as determination of the ploidy levels of individual nuclei, gene expression regulation by nuclear proteins, and maintenance of chromatin structure. The interphase nucleus contains the nuclear envelope associated with the nuclear lamina (Aebi et al. 1986; Heitlinger et al. 1991). In metazoan cells, the major components of the nuclear lamina are intermediate filament proteins called lamin (Goldman et al. 2002), whereas those in the nucleus of plants and yeast are not lamin, which is not present at all, but proteins that form nuclear structures similar to lamina (Mirzayan and Wimmer 1992; Masuda et al. 1997). There have been no reports on the structural proteins of nuclear lamina in filamentous fungi. Preparation of isolated nuclei of filamentous fungi is indispensable in these and other investigations.

Isolation of the nuclei from filamentous fungi requires removing the cell walls and removing the debris of crushed cell walls and other cellular components. In the case of *Neurospora crassa* (Reich and Tsuda 1961; Hsiang and Cole 1973), a cell-wall-less mutant was chosen as the material. In the case of *Aspergillus nidulans*, on the other hand, a wild-type line was used (Gealt et al. 1976), and the purity of nuclei was improved by density gradient centrifugation using Nycodentz (Vagvolgyi and Ferenczy 1991). Thus, most of the established methods for preparing fungal nuclei depend on cell lines, or ultracentrifugation with an expensive polydispersive agent. Hautala et al. (1977) isolated nuclei from a wild-type *N. crassa.* They succeeded in fractionating nuclei using Ludox (W.R. Grace & Co., Columbia, MD, USA), a colloidal silica sol. Using their concept of disrupting cell walls of a wild-type strain and fractionating the nuclei by density gradient centrifugation, we attempted to develop a general method for isolating nuclei from filamentous fungi. To isolate fungal nuclei, we developed an efficient and simple method using *Basidiobolus ranarum* mycelia. The method involves two steps: disruption of cells and fractionation of the nuclei.

Basidiobolus as used in this work has a nucleus approximately 5.0 μm in diameter (Fig. 1), which was easily observed under a microscope. The large nuclei, which are larger than those of fungi reported by Robinow (1963), should be ideal for establishing a method for preparing isolated nuclei from fungi. Besides this property, *Basidiobolus* is a fungus of interest in taxonomy. It had been classified in Entomophthorales (Zygomycota), but recently the classification has been reconsidered (White et al. 2006; Hibbett et al. 2007).

A strain (deposition is now undergoing processing) of *Basidiobolus* was isolated from amphibian excreta collected at Hui-Sun Forest Station, Ren-Ai Xiang, Nantou County, Taiwan, on 11 November 2005 by Y. Degawa and identified as *B. ranarum* Eidam. The mycelia were cultured in 60 ml 20% (w/v) potato exudates and 2% (w/v) dextrose in a 200-ml Erlenmeyer flask at 24°C in the dark.

To prepare isolated nuclei, cell fractionation is needed to disrupt the cells and collect nuclei. In fungi and plants, the first step of cell fraction involves maceration of the cell walls. Principally, this can be achieved by either mechanical disruption (Gealt et al. 1976), enzymatic maceration (Vagvolgyi and Ferenczy 1991), or use of a wall-less mutant

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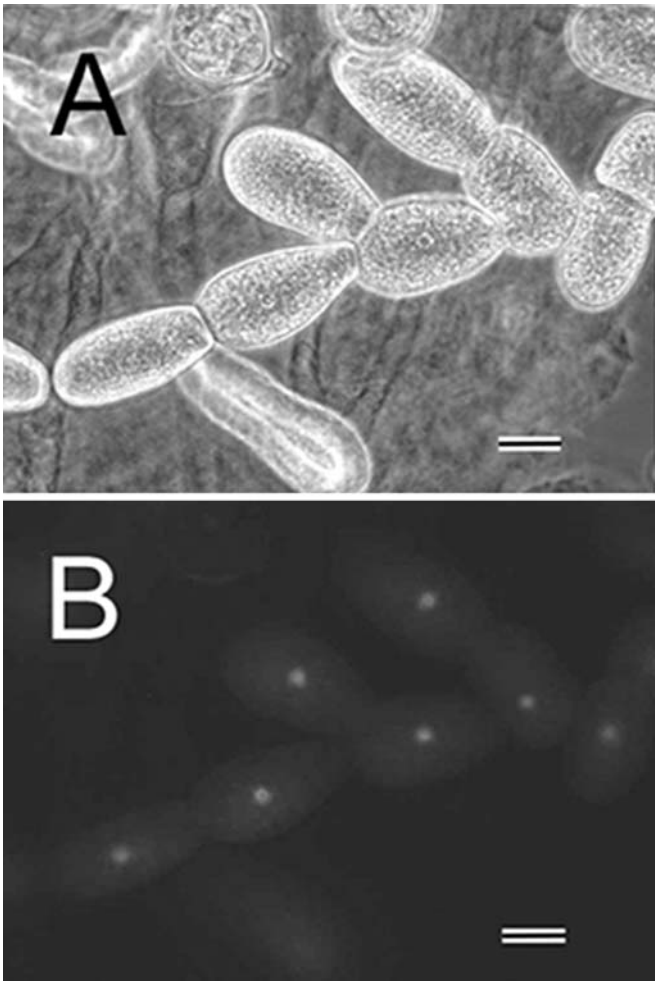

Fig. 1. Mycelia of *Basidiobolus ranarum* in logarithmic growth phase stained with 4',6-diamidino-2-phenylindole (DAPI). **A** Bright-field image. **B** Fluorescent image. The field shown in **B** is the same as that in **A**. *Bars* 20 µm

(Hsiang and Cole 1973). Some methods for fractionating cell nuclei have been reported. Essentially, the methods involved gentle disruption of cells and cell fractionation, mainly by centrifugation. In this work, we compared the efficiency of cell wall maceration of the mycelia of *B. ranarum* between mechanical disruption and enzymatic maceration. Once the mycelia were homogenized, the same method was used to fractionate the cells.

For mechanical disruption, about 8 g (fr. wt.) of mycelia 5 days after inoculation were harvested and washed three times with distilled water. Then they were crushed with liquid nitrogen using a mortar and pestle.

Enzymatic maceration was done using the following procedure. About 8 g (fr. wt.) of mycelia obtained 5 days after inoculation were washed three times with distilled water, and incubated in 0.1 M phosphate buffer (NaH_2PO_4-Na_2HPO_4, pH 7.0) containing 0.2% 2-mercaptoethanol and 40 mM ethylenediaminetetraacetic acid (EDTA) for 20 min at 24°C to help enzymatic maceration. Then, the cells were washed three times with 0.1 M phosphate buffer containing 0.8 M KCl and digested for 1 h with 5 volumes of enzyme solution containing 3% Driserase (Kyowa Hakko, Tokyo, Japan) and 0.5% Cellulase ONOZUKA RS (Yakult, Tokyo, Japan) in the same buffer by gently shaking at room temperature. These macerating enzymes are commercially provided and applicable to other fungi (Asai et al. 1986). The homogenate was used for further fractionation.

Both mechanically disrupted and enzymatically macerated mycelia were homogenized according to the method described by Masuda et al. (1991) with minor modifications. The mycelia were homogenized using a Potter-Elvehjem homogenizer with 10 ml ice-cold 25 mM methanesulfonate (MES, pH 5.8), 5 mM $CaCl_2$, 30 mM KCl, 20 mM NaCl, 300 mM sucrose, 10% (v/v) glycerol, 2% (v/v) Triton X-100, and 2 mM dithiothreitol. After centrifugation at 750 *g* for 15 min, the pellet was resuspended in 25 ml GMC/0.4T (20 mM MES, 30% glycerol, 400 mM sucrose, 10 mM KCl, 10 mM $CaCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, and 0.4% (w/v) Triton X-100) and homogenized by a Teflon homogenizer.

In liquid nitrogen, mycelia of *B. ranarum* were disrupted within several minutes. However, this simple way of homogenizing cells resulted (as shown in Fig. 2A,B) in a large amount of cell debris in the preparation.

The mycelia became fragile when they were incubated in an enzyme solution. The digested mycelia were easy to homogenize using a Potter-Elvehjem homogenizer with a buffer containing a non-ionic detergent. The yield and purity of nuclei were much better than in mechanical homogenization of cells after the cell fractionation process (Fig. 2B,D). When the same amount of mycelia was used, enzymatic maceration reduced the amount of pellets in the cell debris after homogenization compared to that from mechanical disruption. Thus, enzymatic maceration was chosen for breaking down the cell walls.

Organelles can be fractionated by their size, specific gravity, electric charge, and epitopes of the surface. In this work, we fractionated the nuclei based on their size and specific gravity. Size fractionation was the first step of nuclei fractionation. We examined the effects of sieves for removing cell debris. Cell debris was effectively removed when the crude homogenate was filtered through 63-µm and then 22-µm sieves.

Centrifugal force and composition of buffers are the keys in density gradient centrifugation. Centrifugal force was examined by the following procedure. The crude filtrate was layered on 10 ml 50% G/MKC (50% glycerol, 20 mM MES, 10 mM KCl, 10 mM $CaCl_2$) and centrifuged at 1000, 1500, and 2000 *g* for 15 min. After centrifugation, particles > 0.3 µm in diameter and nuclei were counted under a microscope equipped with phase-contrast and epifluorescence optics (DMRB; Leica, Wetzlar, Germany). The nuclei could be distinguished from the other particles by staining with Hoechst 33258. The rates of nuclei per total particles > 0.3 µm obtained by 1000, 1500, and 2000 *g* were 0.40 ± 0.05, 0.72 ± 0.02, and 0.53 ± 0.03, respectively (Table 1). Thus, centrifugation at 1500 *g* was the most effective.

The composition of the media for density gradient centrifugation is also important and many media have been

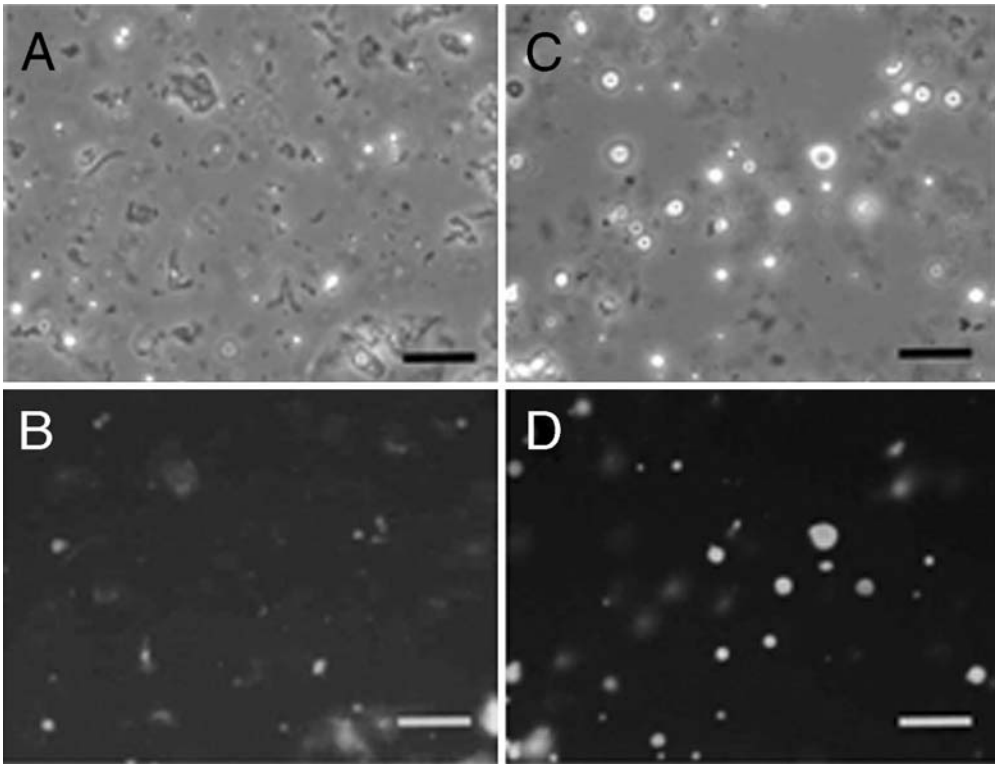

Fig. 2. Isolated nuclei of *Basidiobolus ranarum* stained with Hoechst 33258. **A**, **B** Prepared with mechanical disruption. **C**, **D** Prepared with enzymatic maceration. The same fields were examined using phase-contrast optics (**A**, **C**) or epifluorescence optics (**B**, **D**). Nuclei are indicated with *bright images*. *Bars* 20 μm

Table 1. Purity of isolated nuclei in different conditions

Method/condition	Condition	Rate of nuclei/total particle ± SD[a]
Centrifugal force (g)[b]	1000	0.40 ± 0.05
	1500	0.72 ± 0.02
	2000	0.53 ± 0.03
Percoll concentration (% v/v)[c]	32/48	0.39 ± 0.06
Glycerol concentration (% v/v)[d]	50	0.72 ± 0.02
	40	0.46 ± 0.04

[a] Rate of nuclei in total particles larger than 0.3 μm in diameter; mean values ± SD are shown
[b] The buffer contained 50% glycerol
[c] The buffer contained 30% glycerol
[d] Centrifuged at 1500 *g*

developed for this purpose. For example, sucrose, Ficoll, Percoll, and Metrizamide are widely used in fractionation by centrifugation. Percoll is particularly widely used in nuclei preparation as it has low viscosity and does not affect the osmotic pressure of the medium. On the other hand, our preliminary experiment suggested that glycerol could be effective. Glycerol is not only nontoxic but also reduces mechanical damages to nuclei during isolation. It is thus an ideal compound for density gradient centrifugation. Therefore, we compared the efficiency of Percoll and glycerol. For Percoll density gradient centrifugation, we used the method reported by Masuda et al. (1991) for fractionation of carrot nuclei. We put 10 ml 48% Percoll, 400 mM sucrose, 30% glycerol, 20 mM MES (pH 5.8), 10 mM KCl, 10 mM $CaCl_2$, and 0.2% Triton X-100 in a tube and then added 10 ml buffer of the same composition, except that the Percoll concentration was reduced to 32%. The crude extract of nuclei was loaded on top of the discontinuous Percoll gradient and centrifuged at 8000 *g* for 45 min. Fractionation of nuclei using glycerol was performed as follows. The mycelial extract was loaded on 10 ml 40% or 50% glycerol, 20 mM MES, 10 mM KCl, and 10 mM $CaCl_2$, followed by centrifugation at 1500 *g* for 15 min. The best result was obtained using 50% G/MKC, and the rate of nuclei per total particles > 0.3 μm in this protocol was 0.72 ± 0.02 (see Table 1).

Further observation was performed under a scanning electron microscope (SEM). The nuclei were fixed with 3% (w/v) glutaraldehyde in a 50 mM sodium phosphate buffer (pH 6.7) containing 400 mM sucrose, and postfixed with 2% OsO_4 for 2 h at 4°C. The fixed nuclei were dehydrated through an ethanol series and transferred to *t*-butyl alcohol. The specimens in *t*-butyl alcohol were freeze dried in a Hitachi ES-2030 Freeze Dryer (Tokyo, Japan). The dried specimens were coated with platinum-palladium using a Hitachi E-1030 Ion Sputter Coater (Tokyo, Japan). The nuclei were observed with a Hitachi S-4200 SEM (Tokyo, Japan) operating at 15 kV. The SEM observation revealed that the surface of the isolated nuclei were rough and bumpy (Fig. 3). Although the outer membrane was solubilized by the Triton-X 100 treatment, the shape was conserved well, probably because of the skeletal support by the nuclear matrix. Moreover, observation of more than 10 nuclei under SEM showed the nuclei were spherical with diameters of 1.5–5.0 μm, which was almost the same size as the nuclei before cell fractionation (see Fig. 1).

The recovery of nuclei was evaluated from the amount of DNA from both the initial material and the fractionated nuclei. For quantification, DNA was extracted from the

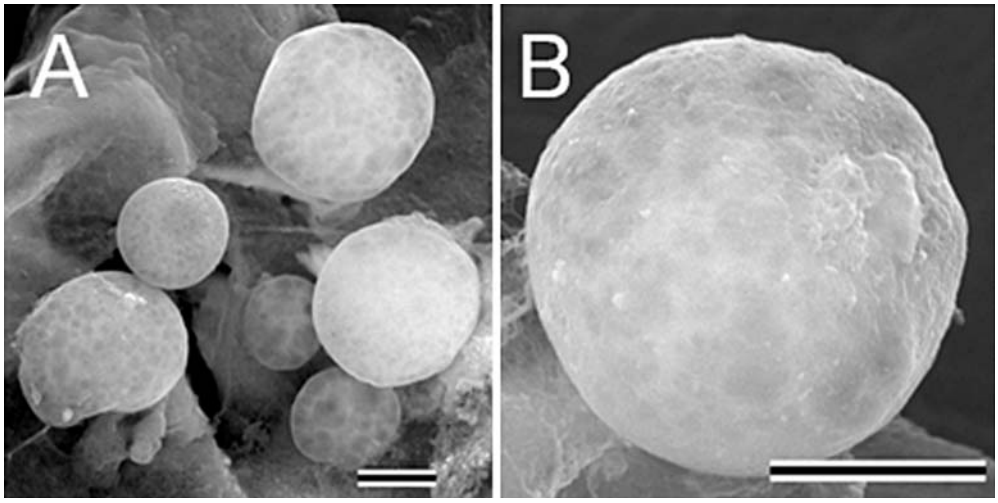

Fig. 3. Scanning electron micrographs of isolated nuclei from *Basidiobolus ranarum*. **A** and **B** are different magnifications. *Bars* 1.5 μm

mycelia and the nuclei according to a modified STS method (Schneider 1946). The DNA concentration was determined using the method of Burton (1956) with minor modifications. The amounts of DNA indicated that the recovery of nuclei was about 10% in our method. Gealt et al. (1976) reported that recovery of nuclei from *Aspergillus nidulans* was 18%. In our method, the recovery of nuclei was not as high as their report. However, our method is quite simple and applicable.

We established a procedure for obtaining a large amount of pure nuclei from the mycelia of *B. ranarum.* First of all, enzymatic digestion of cell walls was essential although it was partial digestion. Filtration through a fine sieve was effective to remove cell debris. Finally, centrifugation using a buffer containing 50% glycerol gave the best results to obtain a pure nuclear fraction. The rate of nuclei per total particles > 0.3 μm was 0.72 ± 0.02. The method established in this work would be useful for studies on filamentous fungal nuclei.

References

Aebi M, Hornig H, Padgett RA, Reiser J, Weissmann C (1986) Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell 47:555–565

Asai T, Okuno T, Matsuura K (1986) Isolation and germination type of protoplasts of spores and hyphae of *Pyricularia oryzae*. Ann Phytopathol Soc Jpn 52:843–849

Burton K (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–322

Gealt MA, Sheir-Neiss G, Morris NR (1976) The isolation of nuclei from the filamentous fungus *Aspergillus nidulans*. J Gen Microbiol 94:204–210

Goldman RD, Gruenbaum Y, Moir RD, Shumaker DK, Spann TP (2002) Nuclear lamins: building blocks of nuclear architecture. Genes Dev 16:533

Hautala JA, Conner BH, Jacobson JW, Patel GL, Giles NH (1977) Isolation and characterization of nuclei from *Neurospora crassa*. J Bacteriol 130:704–713

Heitlinger E, Peter M, Haner M, Lustig A, Aebi U, Nigg EA (1991) Expression of chicken lamin B2 in *Escherichia coli*: characterization of its structure, assembly, and molecular interactions. J Cell Biol 113:485–495

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miądlikowsk J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res 111: 509–547

Hsiang MW, Cole RD (1973) The isolation of histone from *Neurospora crassa*. J Biol Chem 248:2007–2013

Masuda K, Takahashi S, Nomura K, Inoue M (1991) A simple procedure for the isolation of pure nuclei from carrot embryos in synchronized cultures. Plant Cell Rep 10:329–333

Masuda K, Xu ZJ, Takahashi S, Ito A, Ono M, Nomura K, Inoue M (1997) Peripheral framework of carrot cell nucleus contains a novel protein predicted to exhibit a long α-helical domain. Exp Cell Res 232:173–181

Mirzayan C, Wimmer E (1992) Genetic analysis of an NTP-binding motif in poliovirus polypeptide 2C. Virology 189:547–555

Reich E, Tsuda S (1961) Isolation of nuclei of *Neurospora crassa*. Biochim Biophys Acta 53:574–575

Robinow CF (1963) Observations on cell growth, mitosis, and division in the fungus *Basidiobolus ranarum*. J Cell Biol 17:123–152

Schneider WC (1946) Phosphorus compounds in animal tissues. iii. A comparison of methods for the estimation of nucleic acids. J Biol Chem 164:747–751

Vagvolgyi C, Ferenczy L (1991) Isolation of nuclei from *Aspergillus nidulans* protoplasts. FEMS Microbiol Let 82:247–252

White MM, James TY, O'Donnell K, Cafaro MJ, Tanabe Y, Sugiyama J (2006) Phylogeny of the Zygomycota based on nuclear ribosomal sequence data. Mycologia 98:872–884