Author(s): Roberts Andy V.
Title: The Use of Bead Beating to Prepare Suspensions of Nuclei for Flow Cytometry from Fresh Leaves, Herbarium Leaves, Petals and Pollen
Year of publication: 2007
Citation: Roberts A.V. (2007) 'The Use of Bead Beating to Prepare Suspensions of Nuclei for Flow Cytometry from Fresh Leaves, Herbarium Leaves, Petals and Pollen' *Cytometry Part A,* 71A (12) 1039-1044
Link to published version: http://dx.doi.org/10.1002/cyto.a.20486
DOI: 10.1002/cyto.a.20486

Cytometry
PART A
Journal of the International Society for Analytical Cytology

The Use of Bead Beating to Prepare Suspensions of Nuclei for Flow Cytometry from Fresh Leaves, Herbarium Leaves, Petals and Pollen

Andy V. Roberts*

School of Health and Bioscience, University of East London, London E15 4LZ, United Kingdom

Received 21 December 2006; Revision Received 26 July 2007; Accepted 4 October 2007

*Correspondence to: Andy Roberts, School of Health and Bioscience, University of East London, Romford Road, London E15 4LZ, United Kingdom

Email: a.v.roberts@uel.ac.uk

Published online 7 November 2007 in Wiley InterScience (www.interscience.wiley.com)

DOI: 10.1002/cyto.a.20486

• **Abstract**

"Bead beating" is commonly used to release DNA from cells for genomic studies but it was used here to prepare suspensions of plant nuclei for measurement of DNA amounts by flow cytometry. Plant material was placed in 2-ml screw-capped tubes containing beads of zirconia/silica (2.5 mm diameter) or glass (2.5 or 1.0 mm diameter) and 1 ml of lysis buffer. The tubes were mechanically shaken with an FP120 FastPrep Cell Disrupter to release intact nuclei from plant tissue by the impact of the beads. The nuclei were then stained with propidium iodide (PI) and analyzed by flow cytometry. The method was tested using fresh leaves, fresh petals and herbarium leaves of *Rosa canina*, leaves and pollen of *R. rugosa*, and fresh leaves of *Petroselinum crispum*, *Nicotiana tabacum*, and *Allium cepa*. Batches of 12 samples of fresh leaves were prepared, simultaneously, in 45 s by bead beating in the Cell Disrupter. In flow cytometry histograms, nuclei of fresh leaves gave G_1/G_0 peaks with CVs of less than 3.0% and nuclei from fresh petals and herbarium leaves of *R. canina*, and pollen of the generative nuclei of *R. rugosa* gave peaks with coefficients of variation (CVs) of less than 4.0%. DNA amounts estimated from 24-month-old herbarium leaves, using *P. crispum* as an internal standard, were less than those of fresh leaves by a small but significant amount. Suspensions of nuclei can be prepared rapidly and conveniently from a diversity of tissues by bead beating. Exposure of laboratory workers to harmful substances in the lysis buffer is minimized. © 2007 International Society for Analytical Cytology

• **Key terms**

bead beating; fresh leaves; herbarium leaves; petals; pollen; *Allium cepa*; *Nicotiana tabacum*; *Petroselinum crispum*; *Rosa canina*; *Rosa rugosa*

FLOW cytometric analysis of suspensions of plant nuclei stained with fluorochromes is used for ploidy screening, detection of mixoploidy and aneuploidy, cell cycle analysis, assessment of polysomaty, and estimation of genome size (1). Suspensions of nuclei are usually prepared by chopping tissues with a razor blade in lysis buffer (2). The tediousness of chopping led the author to seek an alternative method.

Bead beating is a procedure used to release DNA from the cells of plants (3), animals (4), bacteria (5), and fungi (6). Samples are placed in screw-capped tubes containing beads and are mechanically shaken in a cell disrupter so that the impact of the beads breaks up the cells. The disruptive effect can be varied according to the size and density of the beads, and the speed setting of the shaker. Commonly used beads are 0.1–2.5 mm in diameter and composed of materials of various densities, including of glass (2.5 g ml^{-1}) and zirconia/silica (3.7 g ml^{-1}).

Although flow cytometry could provide a unique insight into DNA content of microspores, it has infrequently been used because of difficulties in preparing suspensions of nuclei. Suspensions of nuclei have been prepared by chopping pollen of *Lilium longiflorum*, *Dendranthema grandiflora*, and *Zea mays* (7), gently squashing pollen of *Rosa* (8) and ultrasonic treatment of pollen of *Brassica napus* and *Triticum*

ISAC
International Society for Analytical Cytology

aestivum (9). These methods involved staining with 4′,6-diamidino-2-phenylindole (DAPI) to give histograms with clear peaks that allowed relative DNA amounts to be assessed. Suspensions of nuclei from herbarium leaves have been prepared by chopping and staining with DAPI to give peaks with low coefficients of variation (CVs) in flow cytometry histograms (10). Fluorometric estimates of DNA amounts are dependable with PI staining but not with DAPI staining (11), so procedures involving PI staining are needed in conjunction with both pollen and herbarium specimens.

This report concerns the effectiveness of bead beating in preparing suspensions of nuclei for flow cytometry. The sources of nuclei were fresh leaves, fresh petals, pollen, and leaves of herbarium specimens.

Materials and Methods

Plant Materials

Fresh leaves and petals were taken from a wild plant of *Rosa canina* L. ($2n = 35$) in Epping Forest, UK. Herbarium specimens from the same plant were prepared 24 months earlier by air drying between sheets of paper in a herbarium press. Pollen was obtained from a garden-grown plant of *Rosa rugosa* Thunb. ($2n = 14$) from which flowers were picked just before anthesis. The sepals and petals were removed, the flowers were left to dry in an open Petri dish on a laboratory bench at ~23°C, and the pollen was then shaken from the flowers and stored in screw-capped tubes at ~23°C. Care was taken to remove anther and filament contaminants from the pollen. *Petroselinum crispum* (Mill.) Nyman "Champion Moss Curled," which was used as a calibration standard, was grown from seed (Thompson and Morgan (UK) Ltd, Ipswich, UK). Leaves of *Nicotiana tabacum* L. were taken from seedlings. Newly expanded leaves of *Allium cepa* L. were taken from commercially obtained bulbs.

Preparation of Suspensions of Nuclei

Suspensions of nuclei were prepared from each sample of leaf or petal material in 1 ml of lysis buffer using an intact piece of tissue of ~7 mg, with or without an equal weight of leaf tissue of *P. crispum*, the calibration standard. *P. crispum* has a 2C DNA amount of 4.46 pg (12). The dried leaves of herbarium specimens of *R. canina* were similarly treated except that 14 mg of tissue was taken, together with 7 mg of *P. crispum* leaves. The lysis buffer (13) contained 0.1 M citric acid and 0.5% Triton X-100 in deionized distilled water. Using the chopping method, the plant material was finely chopped in lysis buffer in a Petri dish (90 mm diameter) with a sharp razor blade for 1–2 min. Using the bead beating method, the plant material and lysis buffer were placed in screw-capped tubes (2 ml, Heather Scientific, Glasgow) along with 10 zirconia/silica beads of 2.5 mm diameter (Heather Scientific, Glasgow) or the same weight (~0.25 g) of glass beads of either 2.5 mm or 1.0 mm diameter. Up to12 such sample tubes were agitated simultaneously in an FP120 FastPrep Cell Disrupter (Savant Instruments, New York). This reciprocating shaker pulverizes cells by multidirectional agitation and can be set at speeds of 4.0–6.5 m s^{-1} (in increments of 0.5 m s^{-1}) for times of 1–45 s. In the experiments reported here, it was set at speeds of either 4.0 or 6.5 m s^{-1} and run for 30 or 45 s. Macerated leaf tissue, prepared either by chopping or bead beating, was filtered through a 30 μm nylon mesh into a test tube (55 × 12 mm). The filtrate (~0.5 ml) was treated with 50 μl of a DNase-free solution of RNase (3 mg l^{-1}; Sigma Plc, Poole) and incubated at 37°C for 30 min. Alternatively, where specified, the DNase-free solution of RNase was omitted or added along with the staining solution referred to below.

A two-stage procedure was used to prepare suspensions of pollen nuclei of *R. rugosa* together with leaf nuclei of the calibration standard, *R. canina*. First, fresh leaf tissue (7 mg) was placed in a screw capped tube (2 ml) along with 10 zirconia/silica beads (2.5 mm diameter) and 1 ml of lysis buffer, and agitated a speed of 5 m s^{-1} for 45 s. The suspension was then filtered and the filtrate was added to pollen (4 mg) in a fresh tube, along with glass beads of 1.0 mm diameter. (1–2 flowers provided sufficient pollen for a single sample of 4 mg.) Lysis buffer was added to bring the total volume to 1 ml. The mixture of leaf and pollen nuclei was then agitated at 4 m s^{-1} for 30 s. The final suspension was then filtered and the filtrate was incubated with RNase at 37°C for 30 min.

Staining of Nuclei and Flow Cytometry

A staining solution was prepared (13) which comprised 11.36 g of Na_2HPO_4, 12 ml of PI stock (1 mg ml^{-1} in water) and 20 ml of 10× stock (100 mM sodium citrate, 250 mM sodium sulphate) made up to 200 ml with deionized distilled water. After incubation of the suspension of nuclei with RNase (where applicable), 0.4 ml of treated filtrate was added to 2 ml of the PI staining solution (giving a final PI concentration of 50 μg ml^{-1}), mixed thoroughly, then incubated at 20–25°C for 20 min. Stained samples were analyzed using a Partec CAIII flow cytometer (Partec GmbH, Munster, Germany) with an argon laser light source (488 nm wavelength), a TK420 dichroic mirror, an OR610 barrier filter, and a 40 × 0.80 quartz objective. The flow cytometry histograms were all ungated and were analyzed automatically by the integral CA3 Partec Quantum Analysis software which automatically set gates around the peaks, provided the means and CVs of the peaks, total number of particles ml^{-1} of suspension, and the proportion of those particles in each peak. The numbers of particles ml^{-1} of the suspension corresponding to the peaks were estimated as the product of the total number of particles and the fraction of particles in the respective peaks. Where internal calibration standards were used, the nuclear DNA amounts of the test species were calculated as the ratio of the channel number of the G_0/G_1 peak of the test species to that of the calibration standard multiplied by the 2C DNA amount of the calibration standard.

Experimental Design and Statistics

Throughout the investigation, four samples were prepared per treatment and three flow cytometric assays were made of each sample. The significances of differences amongst

Table 1. Mean numbers of nuclei ml^{-1} corresponding to the G_1/G_0 peaks and mean CVs of G_1/G_0 peaks in suspensions of nuclei released from *R. canina* leaves by bead beating (speed 6.5 m s^{-1} for 45 s) with beads of zirconia/silica (Z/S) or glass of specified diameter, or by the chopping method

METHOD	MEAN NO. G_1/G_0 NUCLEI ML^{-1} ($\times 10^{-3}$) ± SD	MEAN CV (% ± SD)
Bead beating		
Z/S 2.5 mm diameter	90.3 ± 31.9	2.75 ± 0.26
Glass 2.5 mm diameter	70.1 ± 39.9	2.67 ± 0.28
Glass 1.0 mm diameter	16.5 ± 4.9	2.67 ± 0.37
Chopping	11.9 ± 2.0	2.96 ± 0.27
Significance[a] in F tests		
Among treatments	**	ns
Among samples	***	ns

[a]ns, not significant ($P > 0.05$); *, $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

treatment means and amongst samples within treatments were tested using a nested analysis of variance (ANOVA) (14).

Results

The main peaks observed in all flow cytometry histograms of the nuclei of fresh leaves, fresh petals, and herbarium leaves were of nuclei at the G_0/G_1 stage of the cell cycle. The G_2 peak was either small or undetectable.

The mean densities of nuclei and the mean CVs of G_0/G_1 peaks in suspensions of nuclei prepared from leaves of *R. canina* by bead beating and chopping were measured (Table 1). As no internal standard was used and there were few nuclei of *R. canina* at S or G_2, no significant debris was created that might have led to errors in the quantification of nuclei in the G_0/G_1 peak of *R. canina*. Bead beating was carried out at a speed of 6.5 m s^{-1} for 45 s with three different types of bead: zirconia/silica beads of 2.5 mm diameter, glass beads of 2.5 mm diameter, and glass beads of 1.0 mm diameter. The density of nuclei obtained with zirconia/silica beads of 2.5 mm diameter was highest (90,300 nuclei ml^{-1}), lower with glass beads of 2.5 mm diameter (70,100 nuclei ml^{-1}) and lower still with glass beads of 1.0 mm diameter (16,500 nuclei ml^{-1}). Chopping gave the lowest density of nuclei, 11,900 nuclei ml^{-1}. A nested ANOVA showed that differences were significant both among sample means within treatments ($P < 0.001$) and among treatments ($P < 0.01$). The mean CVs of the G_0/G_1 peaks obtained by the four different procedures were all less than 3.0% and differences were not significant either among samples or among treatments (Table 1). To assess the bead beating method on species with larger DNA amounts, zirconia/silica beads of 2.5 mm diameter were tested at a speed of 6.5 m s^{-1} for 45 s on fresh leaves of *N tabacum* which has a 2C DNA amount of 11.7 pg (15) and *A. cepa* which has a 2C DNA amount of 33.5 pg (16). A mean density of 9,300 nuclei ml^{-1} was obtained for *N. tabacum* and 8,300 nuclei ml^{-1} for *A. cepa* (Table 2), and the mean CVs for both species were less than 2.6% (Table 2).

Table 2. Mean numbers of nuclei ml^{-1} corresponding to the G_1/G_0 peaks and mean CVs of G_1/G_0 peaks in suspensions of nuclei released from fresh leaves of *Nicotiana tabacum* and *Allium cepa* by bead beating with beads of zirconia/silica (2.5 mm diameter) at a speed of 6.5 m s^{-1} for 45 s

SPECIES	MEAN NO. G_1/G_0 NUCLEI ML^{-1} ($\times 10^{-3}$) ± SD	MEAN CV (% ± SD)
Nicotiana tabacum	9.28 ± 2.69	2.56 ± 0.49
Allium cepa	8.32 ± 4.90	2.21 ± 0.43

The DNA amount of fresh leaves of *R. canina* was estimated using fresh leaves of *P. crispum* as an internal calibration standard. Suspensions of nuclei were obtained by bead beating with zirconia/silica beads of 2.5 mm diameter at a speed of 6.5 m s^{-1} for 45 s. Three methods were investigated: RNase was (a) added to the suspension of nuclei in lysis buffer and incubated for 30 min at 37°C before the addition of staining solution (the standard protocol of this paper) (Fig. 1), (b) added at the same time as the staining solution, (c) not added (control). The estimated DNA amounts did not differ significantly either among samples or among treatments (Table 3). The CVs of the G_1/G_0 peaks of *R. canina* did not differ significantly among samples but did among treatments, whereas the CVs of *P. crispum* differed significantly among samples but not among treatments (Table 3). Differences among the mean ratios of *R. canina* to *P. crispum* nuclei in the G_1/G_0 peaks were not significant ($P > 0.05$) among samples or treatments (Table 3).

Bead beating was tested on fresh petals and herbarium leaves (Fig. 2) of *R. canina*, using zirconia/silica beads of 2.5 mm diameter at a speed of 6.5 m s^{-1} for 45 s and *P. crispum* as an internal calibration standard. The mean CVs of G_1/G_0 peaks of *R. canina* petals and herbarium leaves were, respectively, 3.70% and 3.96% (Table 4). The estimated mean DNA amounts for petals (2.93 pg; Table 4) did not differ significantly ($P > 0.05$), in a nested ANOVA from that of fresh leaves of *R. canina* (2.91 pg; Table 3, treatment with incubation

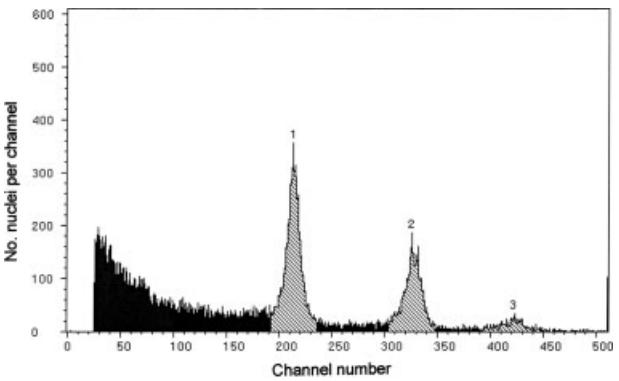

Figure 1. Flow cytometry histogram (ungated) showing the frequency of PI stained nuclei in relation to channel number (linear scale) in a suspension prepared by bead beating leaves of *R. canina* and *P. crispum*. (1, G_1/G_0 peak of *R. canina*, CV 2.0%; 2, G_1/G_0 peak of *P. crispum*, CV 1.3%; 3, G_2 peak of *R. canina*, CV 1.8%).

Table 3. Mean CVs of G_1/G_0 peaks of *R. canina* and *P. crispum* (internal calibration standard), the mean DNA amount of *R. canina* and the mean ratio of the number of nuclei in the G_1/G_0 peaks of *R. canina* to *P. crispum* after bead beating with beads of zirconia/silica (2.5 mm diameter) at a speed 6.5 m s^{-1} for 45 s

RNASE TREATMENTS	MEAN CVs (% ± SD)		DNA AMOUNT OF *R. CANINA* (±SD)	RATIO OF NUMBERS IN G_1/G_0 PEAKS (±SD)
	R. CANINA	*P. CRISPUM*		
A. Before staining	2.77 ± 0.27	2.65 ± 0.33	2.910 ± 0.010	2.01 ± 0.83
B. With stain	2.40 ± 0.23	2.14 ± 0.46	2.910 ± 0.009	2.63 ± 1.19
C. No RNase added	2.67 ± 0.29	2.57 ± 0.55	2.913 ± 0.014	2.67 ± 1.73
Significance[a] in F tests				
Among treatments	**	ns	ns	ns
Among samples	ns	*	ns	***

Three treatments were used: A, RNase added to nuclei in lysis buffer and incubated at 37°C for 30 min before addition of stain; B, RNase added with staining solution; C, no RNase added.

[a] ns, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

before staining). The difference between the estimated mean DNA amounts for herbarium and fresh leaves of *R. canina* were significant ($P < 0.05$) in tests with a nested ANOVA. The ratios of the number of nuclei in G_1/G_0 peaks of petals of *R. canina* to leaves of *P. crispum* (0.59) and herbarium leaves of *R. canina* to fresh leaves of *P. crispum* (0.46) were lower than that of fresh leaves of *R. canina* to fresh leaves of *P. crispum* (2.01; Table 3, treatment with incubation before staining) which indicates that a smaller proportion of nuclei were retrieved from petals and herbarium leaves than from fresh leaves of *R. canina*.

The use of bead beating to release nuclei from pollen was investigated with pollen of *R. rugosa* and fresh leaf tissue of *R. canina* as the calibration standard. In preliminary investigations into the use of bead beating with pollen alone, zirconia/silica beads (2.5 mm diameter) and glass beads (1.0 and 2.5 mm diameter) were tested at speeds of 4 or 6.5 m s^{-1} for 30 or 45 s. When the least rigorous of these treatments was applied (glass beads of 1 mm diameter at 4 m s^{-1} for 30 s), two peaks were observed, one peak having twice the fluorescence intensity of the other. The area of the smaller peak was diminished, relative to the larger peak, when more rigorous protocols were used. When combined samples of pollen and leaves were prepared by the two-stage procedure described in Materials and Methods, the two pollen peaks and G_1/G_0 peak of the *R. canina* leaf could be distinguished (Fig. 3). The peaks with nuclei of higher fluorescence intensity had lower CVs than the peaks with lower fluorescence intensity and were used for the estimation of DNA amounts. The DNA amount of the pollen peak with the higher fluorescence intensity was 1.117 pg and did not differ significantly ($P > 0.05$), in a nested ANOVA, from that of nuclei from fresh leaf tissue of *R. rugosa* (1.125 pg) (Table 5). The ratio of the numbers of nuclei in the *R. rugosa* pollen peak of higher fluorescence intensity to the G_1/G_0 peak of *R. canina* was 1.02, and the ratio of the G_1/G_0 peaks of *R. rugosa* and *R. canina* leaves was 2.75. Less pollen (4 mg) than leaf material (7 mg) was used, so the numbers of pollen nuclei of higher fluorescence intensity and leaf nuclei of *R. rugosa* released per unit weight tissue were similar.

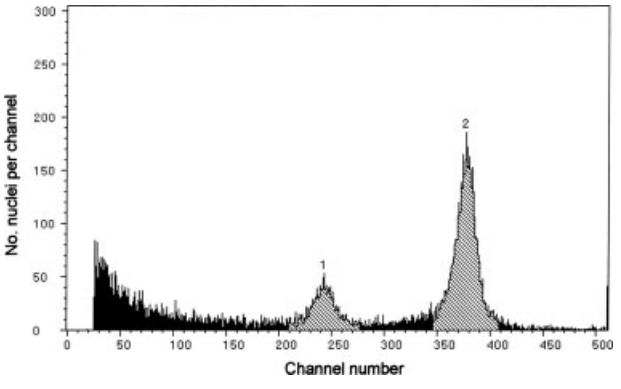

Figure 2. Flow cytometry histogram (ungated) showing the frequency of PI stained nuclei in relation to channel number (linear scale) in a suspension prepared by bead beating herbarium leaves (2 years old) of *R. canina* with fresh leaves of *P. crispum*. (1, G_1/G_0 peak of *R. canina*, CV, 3.2%; 2, G_1/G_0 peak of *P. crispum*, CV, 1.9%).

Discussion

In tests on leaves of *R. canina* involving bead beating with three different types of bead and chopping (Table 1), the mean densities of nuclei differed significantly among samples within treatments and standard deviations (SD) were large, probably because of small differences in the weighed amounts of leaf material used for sample preparation. However, above this level of variation, differences among treatments were also significant ($P < 0.01$). The densities of nuclei were greater in the treatment with zirconia/silica beads of 2.5 mm diameter than with glass beads of 2.5 mm diameter. This was expected because zirconia/silica is denser than glass and the energy of impact of the beads was correspondingly greater. Likewise, the greater concentration of nuclei prepared using glass beads of 2.5 mm diameter than the lighter glass beads of 1.0 mm diameter was expected. The density of nuclei produced by beating with zirconia/silica beads of 2.5 mm diameter was greater, by nearly 8-fold, than by chopping which indicates that the lysis

Table 4. Mean CVs of the G_1/G_0 peaks of *R. canina* and *P. crispum*, mean estimated DNA amounts of *R. canina* and the mean ratios of the numbers of nuclei in the G_1/G_0 peaks of *R. canina* to *P. crispum* after bead beating fresh petals or herbarium leaves of *R. canina* together with *P. crispum* (internal calibration standard)

	MEAN CVS (% ± SD)			
TEST MATERIAL OF *R. CANINA*	*R. CANINA*	*P. CRISPUM*	DNA AMOUNT OF *R. CANINA* (PG ± SD)	RATIO OF NUMBERS IN G_1/G_0 PEAKS (± SD)
Fresh petals	3.70 ± 0.62	2.49 ± 0.35	2.93 ± 0.03	0.59 ± 0.17
Herbarium leaves	3.96 ± 0.38	2.23 ± 0.32	2.86 ± 0.04	0.46 ± 0.12

Bead beating was carried out with zirconia/silica beads (diameter 2.5 mm) at a speed of 6.5 for 45 s.

of the leaf material was more efficient. The CVs of the G_1/G_0 peaks did not differ significantly between treatments, indicating that the density of nuclei did not affect the precision with which the fluorescence intensity of the peaks could be measured. Bead beating was also tested on *N. tabacum* (11.7 pg) and *A. cepa* (33.5 pg) which have larger DNA amounts than the other species investigated. The mean densities of leaf nuclei obtained from *N. tabacum* and *A. cepa* were lower than in *R. canina*, probably reflecting a smaller number of nuclei per unit weight of leaf tissue. The mean CVs were lower than 2.6 in both *N. tabacum* and *A. cepa* (Table 2), indicating that bead beating is a suitable procedure for preparing suspensions of nuclei in species with large genomes.

To maximize the time-saving opportunities provided by bead beating, it would be desirable to avoid incubating the suspension of nuclei in lysis buffer with RNase as a separate stage before staining. Alternative procedures that do not involve separate incubation have been adopted by other authors (e.g. Ref. 17). When the effects of different RNase protocols were studied in combined suspensions of *R. canina* and *P. crispum* Table 3), differences in the estimated DNA amounts of *R. canina* were not significant ($P > 0.05$) among samples or among treatments. This supports the adoption of the faster protocol in which RNase was added with the staining solution; however, as no significance difference was found among treatments that included samples that were not treated with RNase, the adoption of the faster protocol requires confirmation with other species. Differences among the mean CVs of *R. canina* were significant among treatments ($P < 0.01$). The mean CV was greatest in the treatment that involved incubation at 37°C for 30 min, which suggests that this incubation may have had a negative effect on the uniformity of subsequent staining.

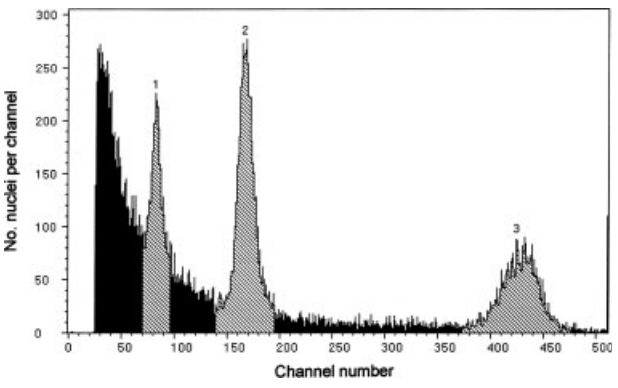

Figure 3. Flow cytometry histogram (ungated) showing the frequency of PI stained nuclei in relation to channel number (linear scale) in a suspension prepared by bead beating pollen of *R. rugosa* and leaves of *R. canina*. (1, peak of vegetative nuclei of *R. rugosa* at G_1/G_0, CV 5.7%; 2, peak of generative nuclei of *R. rugosa* at G_2, CV 3.7%; 3, G_1/G_0 peak of *R. canina*, CV 3.9%).

The mean ratio of numbers of nuclei in the G_1/G_0 peaks of *R. canina* petals to *P. crispum* leaves (Table 4) was lower than that for fresh leaves of *R. canina* to leaves of *P. crispum* (Table 3) which indicates that relatively fewer nuclei were retrieved from fresh petals than fresh leaves of *R. canina*. However, the estimated DNA amount of 2.93 pg did not differ significantly in a nested ANOVA ($P > 0.05$) from the estimate of 2.91 pg based on fresh leaves of *R. canina* (Table 3). The petals of *R. canina* were white with shades of pink but cytosolic components can interfere with fluorochrome staining (18, 19) and might, therefore, affect the measurement of DNA amounts in strongly pigmented petals. The main interest in the use of petals is likely to be in species with leaves that are tough or small, from which it is not easy to obtain good suspensions.

As with petals, ratios of numbers of nuclei in the G_1/G_0 peaks of test species and standard indicate that fewer nuclei were obtained from 24-month-old herbarium leaves than fresh leaves of *R. canina*. Although the mean CV of herbarium leaves of *R. canina* was 3.96% and suitable for measurement of DNA amounts, the estimated DNA amount (2.86 pg) was significantly lower ($P < 0.05$) than that of fresh leaves (2.91 pg) and may indicate a time-related degradation of the DNA. However, these results support the view that flow cytometry of herbarium specimens could have interesting applications in plant systematics provided that due account is taken of any changes in apparent DNA amount that might occur with time (10).

Pollen grains are bicellular at anthesis in *Rosa*, each containing a vegetative cell and a generative cell that divides into two sperm cells ~24 h after pollen germination (8). Prior to this division, the generative nucleus must progress to the G_2 stage of the cell cycle. Therefore, at anthesis, the pollen contains a haploid generative nucleus at the G_2 stage of the cell cycle and a haploid vegetative nucleus at the G_1/G_0 stage. The presence of nuclei of two types, one with twice the fluorescence intensity of the other, was confirmed in *Rosa* by flow cytometry of DAPI stained nuclei released by crushing the pollen (8). Likewise, bicellular pollen in *Lilium longiflorum* was shown to contain a generative cell with twice the DNA amount

Table 5. Mean CVs of G_1/G_0 peaks *R. rugosa* leaves, G_2 peaks of R. rugosa pollen, and G_1/G_0 peaks of *R. canina* leaves, DNA amounts of *R. rugosa* and ratio of numbers of nuclei of *R. rugosa* to *R. canina* in the measured peaks after bead beating leaf tissue or fresh pollen of *R. rugosa*, with leaves of *R. canina* (internal calibration standard)

	MEAN CVs (% ± SD)			
TISSUE OF *R. RUGOSA*	*R. RUGOSA*	*P. CRISPUM*	DNA AMOUNT OF *R. RUGOSA* (PG ± SD)	RATIO OF NUMBERS OF NUCLEI (± SD)
Leaves	3.58 ± 0.33	2.68 ± 0.37	1.125 ± 0.003	2.73 ± 0.04
Pollen	3.92 ± 0.47	2.95 ± 0.46	1.117 ± 0.010	1.02 ± 0.33

of the vegetative cell (7). In the present investigation, the pollen peak of higher fluorescence intensity contained nuclei with the same amount of DNA as diploid somatic nuclei at G_1/G_0 and represents haploid, generative nuclei at G_2. The less well defined peak, with half the amount of DNA, represents haploid vegetative nuclei at G_1/G_0. The vegetative nuclei were more easily damaged during rigorous bead beating, possibly because of their larger size (8). Previously published methods of preparing pollen nuclei for flow cytometry have used DAPI as the fluorochrome which is unsuitable for absolute measurement of DNA amounts (11). One likely application of this method will be to establish variations in DNA amount amongst microspores produced by hybrid plants.

The transfer of samples of fresh leaves in good condition from remote locations to the laboratory for analysis by flow cytometry can be problematic (10). For some species, the collection of pollen (which has a long shelf life in many species) or dehydrated leaf specimens prepared as for herbarium specimens might be useful alternatives to fresh leaves.

Bead beating with the FastPrep Cell Disrupter enabled suspensions of nuclei to be prepared simultaneously from 12 samples by agitating for 45 s, whereas 1–2 min is required to prepare a single sample by the chopping method. In a normal working day, the author was able to prepare, stain, and analyze suspensions of up to 36 leaf samples and estimate DNA amounts in three replicates per sample by flow cytometry. In the chopping method, the gloved hand of the experimenter is in contact with harmful substances in the nuclei isolation buffer. The more remote handing of the isolation buffer in the bead beating method reduces this potential hazard. The versatility of the bead beating method was demonstrated with suspensions of nuclei prepared from fresh leaves of species that range in 2C DNA amounts from 1.13 pg (*R. rugosa*) to 33.5 pg (*A. cepa*), suspensions of nuclei from fresh petals and dry of herbarium leaves of *R. canina*, and pollen of *R. rugosa*. The screw-capped tubes can be reused indefinitely and the beads can be reused ~10 times (http://www.biospec.com/Beads.htm) without appreciable wear, so the only significant additional cost would be the purchase of a cell disrupter by those laboratories that do not already have access to one.

Acknowledgments

The author thanks Dr. I. Leitch, Royal Botanic Gardens, Kew, UK and Dr. J. Dolezel of the Institute of Experimental Botany, Olomouc, Czech Republic for their helpful comments.

Literature Cited

1. Dolezel J, Bartos J. Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot 2005;95:99–110.
2. Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. Rapid flow cytometric analysis of the cell-cycle in intact plant-tissues. Science 1983;220:1049–1051.
3. Haymes KM, Ibrahim IA, Mischke S, Scott DL, Saunders JA. Rapid isolation of DNA from chocolate and date palm tree crops. J Agric Food Chem 2004;52:5456–5462.
4. Harmon AF, Zarlenga DS, Hildreth MB. Improved methods for isolating DNA from *Ostertagia ostertagi* eggs in cattle feces. Vet Parasitol 2006;135:297–302.
5. de Lipthay JR, Enzinger C, Johnsen K, Aamand J, Sorensen SJ. Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. Soil Biol Biochem 2004;36:1607–1614.
6. Borneman J, Hartin RJ. PCR primers that amplify fungal rRNA genes from environmental samples. Appl Environ Microb 2000;66:4356–4360.
7. Bino RJ, Tuyl JMV, de Vries JN. Flow cytometric determination of relative nuclear DNA contents in bicellulate and tricellulate pollen. Ann Bot 1990;65:3–5.
8. Jacob Y, Priol V, Ferrero F, Coudret A, Sallanon H. Fluorescent staining of roses pollen tubes and nuclei by microscopy and flow cytometric analysis. Acta Hortic 2001;547:383–385.
9. Pan G, Zhou YM, Fowke LC, Wang H. An efficient method for flow cytometric analysis of pollen and detection of 2n nuclei in *Brassica napus* pollen. Plant Cell Rep 2004;23:196–202.
10. Suda J, Travnicek P. Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry - new prospects for plant research. Cytometry Part A 2006;69A:273–280.
11. Doležel J, Sgorbati S, Lucretti S. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol Plant 1992;85:625–631.
12. Yokoya K, Roberts AV, Mottley J, Lewis R, Brandham PE. Nuclear DNA amounts in roses. Ann Bot 2000;85:557–561.
13. Hanson L, Boyd A, Johnson MAT, Bennett MD. First nuclear DNA C-values for 18 Eudicot families. Ann Bot 2005;96:1315–1320.
14. Sokal RR, Rolfe FJ. Biometry, 3rd ed. New York: WH Freeman and Company; 1995.
15. Narayan RKJ. Nuclear DNA changes, genome differentiation and evolution in *Nicotiana* (Solanaceae). Plant Syst Evol 1987;175:161–180.
16. Hof JV. Relationship between mitotic cell cycle duration, S period duration and average rate of DNA synthesis in root meristem cells of several plants. Exp Cell Res 1995;39:48–58.
17. Doležel J, Doleželová M, Novák FJ. Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). Physiol Plantarum 1994;36:351–357.
18. Noirot M, Barre P, Louarn J, Duperray C, Hamon S. Nucleus-cytosol interactions—A source of stoichiometric error in flow cytometric estimation of nuclear DNA content in plants. Ann Bot 2000;86:309–316.
19. Noirot M, Barre P, Louarn J, Duperray C, Hamon S. Consequences of stoichiometric error on nuclear DNA content evaluation in *Coffea liberica var. dewevrei* using DAPI and propidium iodide. Ann Bot 2002;89:385–389.