One-Step Transmission E letron M icroscopy of D eoxyribonucleic A cid

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INTRODUCTION

A one-step release-spreading method has been developed with the aim of producing the most conservative and fastest electron microscope screening of an organelle's genomic DNA [¹]. The method is based on simultaneous organelle lysis and DNA release-spreading by a detergent-high salt-ethidium bromide-cytochrome C suspending solution (DSEC) capable of spreading, at its water-air interface, the organelle's disassembled molecules. The molecules so spread are prone to absorb and counter-stain by acidified uranyl acetate (UO₂) after touching the film-coated electron microscope grid [¹].

The method, assayed on a variety of isotonically and hypotonically treated algal organelles, or isolated algal DNAs, has proved to be a very efficient size and heterogeneity spectral indicator especially suited for resuming and screening DNA sub-heteromeres in subliminal amounts [¹,²]. Its proposed use as a fast universal genomic screener, particularly suited for the diagnosis of human mt-DNA pathogenic deletions [³-⁵], is supported here through the cumulative discussion of previous results [¹] and present findings obtained by a refined experimental procedure. The results referring to isotonically or hypotonically treated Acetabularia (A) mitochondria will be detailed in this treatise that document the full power of the method.

THE REFINED PROCEDURE

The improvement of a previous procedure [¹] has been possible through the reformulation of the DSEC composition and use of an unconventional absorption sequence. The reformulated DSEC (DRSS) is made up of the following ingredients which if put in the indicated range of ad hoc adjustable concentrations 10⁻⁴⁻¹⁰⁻¹⁰⁴ M Tris-phosphate-EDTA buffer titrated to pH 8-9 with potassium hydroxide (KOH); 0-0.1 M potassium and calcium chlorides (KCl, CaCl₂); 3-4 μg/ml ethidium bromide (EtBr); 0.05-0.1% (w/v) cyanochrome C (CytC); 0.2-0.3 mg/ml of 4000-8000 polyethylene glycol (PEG); 0.05-0.1% (w/v) sodium deoxocholate (NaDOC); 0.1-0.2 mg/ml Brij 58. On occasion, DRSS may be conveniently enriched by: 0.04-0.1 M monoacidic potassium phosphate (K₂HPO₄); 0.05-0.1% (w/v) malic acid; 10⁻³⁻¹⁰⁻¹⁰⁴ M ammonium acetate; 10⁻¹⁻¹⁰⁻¹⁰⁵ M Triston X-100; 10⁻⁴⁻¹⁰⁻⁶ M octyl-β-D-glucopyranoside (OGP); and 1-3% (w/v) spermonic acid, which together contribute in enhancing DNA's contrast and increasing stability of its supporting film.

DNA samples, either free molecules or genomes encased within the matrix of isotonically or hypotonically treated cytoplasmic organelles are diluted in 0.1-1.0 ml of DNA-preserving solution at concentrations which as previously shown [¹], may range from 10⁻²⁻¹⁰⁻¹³ Daltons/ml or 1⁻¹⁰⁻¹⁰⁻¹⁴ individuals/ml. The diluted samples are then simply mixed in about 80 ml sterile DRSS in a 10cm Petri dish and left to stand at will under the dish cover. At this point DNA testing may be performed by addition of appropriate probes to the DRSS DNA-spreading suspension.

DNA harvesting by simply touching the 75 cm² DRSS sample surface with a coated EM grid may start a few (5-10) minutes after the sample's dilution or manipulation. However because of the sterility provided by the dish cover, it can continue for days until the apparent exhaustion of the sample, given the time-insensitive efficiency of DRSS and the indefinite stability of its DNA monolayer [¹].

The DRSS-spread DNA monolayers manifest differential absorption and staining behaviour with regard to the ground substance of the absorbing film. In fact, they stick promptly to the touching carbon film and can then be counterstained by subsequent immersion for 30-60 sec in an acetone solution containing 5x10⁻⁴ M uranyl acetate, 2% methanol, 2% ethanol and 0.01-0.1% formaldehyde (acidified UO₂), followed by dehydration in 95% ethanol and hexane drying (C-DNA; Fig 1a). On the other hand, the DNA monolayers neither absorb nor contrast on identically processed Formvar-silicon oxide (SO/SO₃) coated grids (FSS) (Touzart and Matignon, Vitry sur Seine, Paris, France) unless the DNA-coated grids are rubbed dry against the freshly cleaved surface of mica, before UO₂, contrast- and post-stain drying (FSSM-DNA; Fig 1b).

Neither improvements of C-DNA quality by addition of appropriate probes to the DRSS DNA monolayer have been proved.

FEATURES AND EFFICIENCY OF THE REFINED METHOD

Both the C- and the FSSM-DNAs from DRSS-lysed Acetabularia mitochondria are in the form of mini- (0.1 to1.0µm long) to maxi- (1.1 to 9.23 µm long) circular, 10-12 nm thick, nearly round and territorially isolated molecules. By both ultrastructural criteria and control experiments, these were unequivocally identified as fully relaxed covalently closed DNA circles (Fig 1a, b).

Both the maxi- and mini- circles of DNA indifferentially absorb on either carbon or SO/SO₃-coated grids, contrary to what was expected. The C-DNA quality by addition of mica-drying of the DNA-coated FSSM has ever been observed.

MICROSCOPY AND ANALYSIS • MARCH 2002

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gest. These, and analogous observations on linear DNAs [1 and unpublished], exclude involvement of any form or size factors regulating DNA absorption under the conditions of the one-step procedure.

The DNA circles on carbon were often in touch with background 0.2-3µm kinked RNAs and 1x3µm globular or fenestrated matrix and membrane remnants (Fig 1a); on FSSM the circles stood out because they were much better contrasted against a much cleaner background (Fig 1b).

Under either random or exhaustive DRSS-DNA monolayer sampling on carbon or FSSM neither overlapping nor juxtaposing DNAs were ever observed.

Similar results were obtained for Acetabularia mt-DNAs at 10⁸ to 10¹³ Daltons/ml (not shown) and organelles at 1 to 10⁴/ml (Fig. 1a, b). These figures are converted into occupied areas (occupancy) by taking into account a conversion factor of 1.9x10⁶ Daltons/µm DNA [1,6,8] plus the ~120µm² of the mean A mt-DNA circle, some 40µm long (Fig 2), and the 3 mm² occupancy of the mean A mitochondrial genome [1,6], the above observation concerns samples exceeding 10¹⁰ to 3 times the 75cm² spreading surface but in no case affecting territorial isolation of spread DNA molecules.

All the evidence indicates that DRSS release-spread DNA molecules neither overcrowd nor overlap on the spreading surface, and when exceeding its full occupancy, randomly fluctuate in the bulk DRSS orderly replacing their spread partners harvested from the surface. Both carbon and FSSM DNAs scatter at the chosen 0.1µm precision limit intervals in a continuous size distribution strongly skewed around the 0.1-1.3µm modal peaks (Fig 2). This pattern, which suggests intramolecular recombination as the source of Acetabularia mt-DNA heterogeneity [1], closely overlaps the size distribution of the free molecules and stable supramolecular aggregates of DNA extruded from A mitochondria by the osmotic shock technique [6]. This indicates that the DRSS is capable of conservative DNA disentanglement and dissociation from in-vivo protein, and possibly RNA, binding constraints [1,6,8] which contribute to the stability of the osmotically resistant protein-DNA [6] aggregates. Adding to the above are the punctual and selective rescuing and preservation of such rare and unstable functional intermediates as the approximately 1% replicating [1] and 10% recombining DNA's retrieved in carbon and FSSM of DRSS-spread A mitochondria or phenol-extracted A mt-DNAs (Mazza in preparation). Neither the DRSS chemicals nor the pH can be freely changed if the efficiency and prerogatives of the one-step method are to be preserved. Only DRSS neutralisation or up to pH 5 acidification seems to be compatible with

Figure 1:
Acetabularia mitochondrial DNA release-spread by the DRSS method.
(a) Montage of images of concentrically arranged maxi DNA (72.4; 66.2; 35.4µm) spread on a noisy heterologously charged carbon film.
(b) Mini (0.4-1.4 µm) circular DNA on the DNA-selecting, electron translucent amorphous silica of silicon oxide-coated grids rubbed dry against mica (FSSM). Scale bars = 1.5µm
the method although leading to nucleoid-like DNA reaggregation and exclusive carbon absorption [1]. On the other hand, the relative proportions of DRSS chemicals seem to need tentative adjustment within the range of concentrations previously indicated, in as yet a priori undefined relation with the variable amounts or compositional patterns of the heterogeneous test materials.

The above findings identifying the critical role of the pH, cations (organic and inorganic), detergent and DNA elastic status as regulators of assembly/disassembly equilibrium and surface spreading capacity, indicate number and types of physicochemical parameters heuristically testable for the purpose of understanding the mechanism and patterns of functional DNA-protein interaction in living organelles. This, together with the DRSS-DNA compatibility and the possibility of straight-on experimental testing, makes both the strength point and the prerogative of the one-step procedure conveniently assayable and adjustable under whatever experimental conditions [7] devised to rescuing quiescent or functionally stimulated cells. The above indicates that the DRSS method does not definitely destroy the functional interactions of DNA-proteins and may permit highly conservative electron microscope analysis of in-vivo like morpho-functional repatterning of DNA through pertinent chemical or enzymatic [1] testing of DRSS-suspended samples. The left-kurtotic (0.1-1.3µm), 0.4µm modal skewed population of the approximately 1700 DNA circulars of Acetabularia mitochondria under the condition of one-step release-spreading electron microscopy. The 0.1µm size intervals reflect the chosen measurement’s standard precision limit of ±0.09µm [1].

REFERENCES

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