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# Improved Preservation of HeLa Cells by Sequential Chemical Addition During Microwave- Assisted Freeze Substitution

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High pressure freezing (HPF) has become the preferred method of preparation for many biological specimens, allowing near native state preservation of structures often lost or distorted during conventional chemical fixation. Adequate HPF of cultured mammalian cells, however, is not easily achieved. Freeze related damage and poor membrane contrast are common problems. Groups have shown addition of cryo-protectants such as 10% BSA in freeze media, and addition of 5% water in freeze substitution media can improve the freezing and membrane contrast respectively [1,2]. Many protocols simultaneously combine fixatives into one step during freeze substitution, while others have shown addition of fixatives in sequential steps may offer advantages [3]. In this study we sought to optimize a freeze substitution method for HeLa cells, a commonly used model cell line in human pathogen investigations.

HeLa cells were briefly fixed with 2.5% glutaraldehyde to mimic deactivation conditions of pathogens typically used in our studies. Cells were then washed with HBSS, and resuspended in 10% BSA/HBSS, aliquoted into membrane carriers and high pressure frozen in the Leica EMPACT 2. Using microwave-assisted freeze substitution (MWFS) methods developed by our lab, freeze substitution was accomplished using a Ted Pella microwave oven, greatly reducing the processing time from days or even weeks to less than one day. Briefly, crushed dry ice was placed in a styrofoam container and 5 ml glass beakers were inserted and surrounded with dry ice. Frozen specimens were placed at the bottom of each beaker, and pre-cooled freeze substitution media was exchanged as described in Tables 1 & 2. Specimens were infiltrated and embedded in Spurr's resin and polymerized overnight at 68°C. Blocks were sectioned on a Leica EM UC6 microtome, viewed on a Hitachi H-7500 transmission electron microscope, and images were collected with a bottom mount XR-100 AMT digital camera system.

As shown in Figure 1, sequential addition of fixatives showed significant improvement in overall cell architecture. Freeze damage appeared minimal, cytoplasm less extracted, and membranes intact and well contrasted (C & D) compared to specimens with simultaneous addition of fixatives (A & B). This method demonstrates effective methods of freeze substitution for low temperature processing of cells in suspension. Future studies including alternative fixation reagents, varied chemical concentrations, variable microwave time, wattage, and thermal settings may offer further optimization for some cells and tissues.

[1] KL McDonald et al., *Electron Microscopy: Methods and Protocols.*2007 February
[2] P. Walther and A. Ziegler, *Journal of Microscopy.* 2002 October
[3] A. Schlegel et al., *Journal of Virology.* 1996 October
[4] Cells were provided by Drs. George Belov and Ellie Ehrenfeld.

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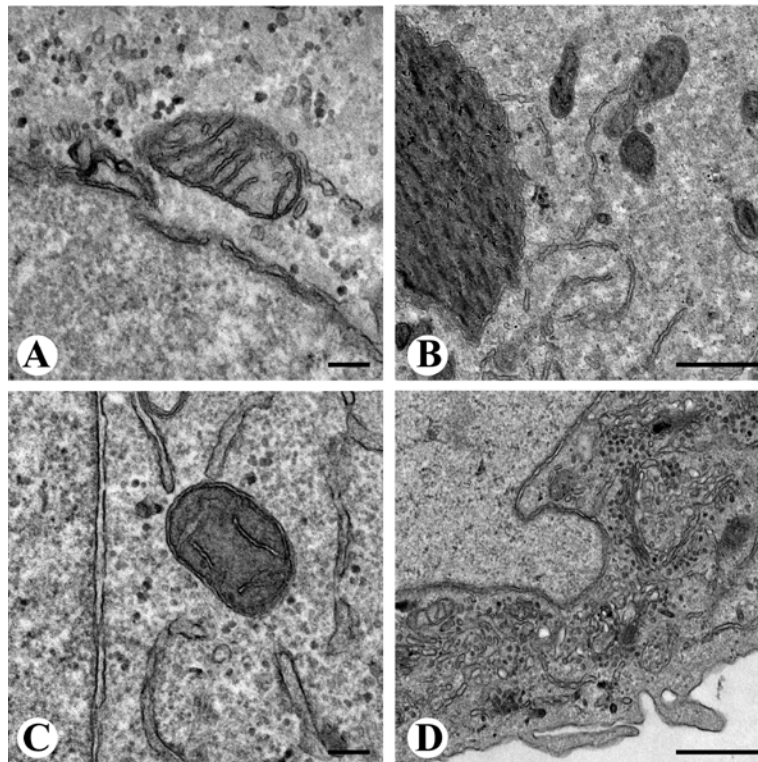


**Figure 1:** This panel shows improved structural preservation of cytoplasmic and membrane components by sequential addition of chemicals (C&D) compared to simultaneous addition of chemicals (A&B). Scale Bar (A&C) = 100nm, (B&D) = 500nm.

**Table 1:** Chemical concentrations and microwave conditions used for simultaneous chemical method

| **Simultaneous Chemical Method** | | | | |
|---|---|---|---|---|
| *Chemical* | *Time* | *Watts* | *Vacuum* | *Temp (approx)* |
| 1% Osmium Tetroxide/ 0.1% Uranyl Acetate | 8 X (2min on – 2min off – 2min on) | 250 | No | -60 to -78°C |
| Wash in Acetone | 3 x 45 Seconds | 250 | No | -60 to -78°C |
| 1:3 Spurr's Resin:Acetone | 5min on – 5min off – 5min on | 250 | Yes | RT |
| 1:1 Spurr's Resin:Acetone | 5min on – 5min off – 5min on | 250 | Yes | RT |
| 3:1 Spurr's Resin:Acetone | 5min on – 5min off – 5min on | 250 | Yes | RT |
| 100% Spurr's Resin | 5min on – 5min off – 5min on | 250 | Yes | RT |

**Table 2:** Chemical concentrations and microwave conditions used for sequential chemical method

| **Sequential Chemical Method** | | | | |
|---|---|---|---|---|
| *Chemical* | *Time* | *Watts* | *Vacuum* | *Temp (approx)* |
| 1% Osmium Tetroxide | 8 X (2min on – 2min off – 2min on) | 250 | No | -60 to -78°C |
| Wash in Acetone | 3 x 45 Seconds | 250 | No | -60 to -78°C |
| 1% Tannic Acid | 8 X (2min on – 2min off – 2min on) | 250 | No | -60 to -78°C |
| Wash in Acetone | 3 x 45 Seconds | 250 | No | -60 to -78°C |
| 1% Uranyl Acetone | 8 X (2min on – 2min off – 2min on) | 250 | No | -60 to -78°C |
| Wash in Acetone | 3 x 45 Seconds | 250 | No | -60 to -78°C |
| 1:3 Spurr's Resin:Acetone | 5min on – 5min off – 5min on | 250 | Yes | RT |
| 1:1 Spurr's Resin:Acetone | 5min on – 5min off – 5min on | 250 | Yes | RT |
| 3:1 Spurr's Resin:Acetone | 5min on – 5min off – 5min on | 250 | Yes | RT |
| 100% Spurr's Resin | 5min on – 5min off – 5min on | 250 | Yes | RT |

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