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Computer Quantification of Intracellular Gold Particles

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ABSTRACT

We describe the use of image software programs available for both PC and Macintosh[®] computers to quantify the accumulation and distribution of gold-labeled constructs within two-dimensional cell sections. The compartmentalization of a biotinylated-peptide was visualized in radiation-induced fibrosarcoma cells by transmission electron microscopy, using a gold particle-streptavidin conjugate. This study illustrates the ease of tabulating gold particles observed in scanned electron micrographs, using Adobe[®] Photoshop[®] in conjunction with the public domain NIH Image program (Version 1.61). Quantitative information regarding the localization of molecules inside cells is crucial in defining their sites of action and in developing more effective therapeutic agents.

INTRODUCTION

Estimating gold particle accumulation within individual cells is often a laborious and time-consuming procedure. All particles within the two-dimensional (2-D) cross section of a cell must be counted to accurately comment

on the distribution of a particular molecule inside cells.

To assess if we could tabulate the number of gold particles present in cell sections using software compatible with Apple Macintosh[®] computers, we examined electron micrographs of radiation-induced fibrosarcoma (RIF-1) cells treated with a biotinylated peptide construct and revealed them using gold-labeled streptavidin. The nonuniform distribution of gold particles was clearly observed, suggesting that the biotinylated peptide was confined to discrete vesicular compartments of these cells. Endocytic uptake and nuclear targeting of the biotinylated construct has been described elsewhere (6). It is clear that the analysis of gold-particle distribution and accumulation within a 2-D section of a cell serves as a useful indication of the targeting efficiency of this construct.

This report introduces a protocol using imaging software for establishing the quantity or density of gold particles within a cell and subcellular regions, which is faster and more accurate than manual procedures. This protocol permits the quantitative analysis of any discernable entities present in confocal images and electron micrographs.

MATERIALS AND METHODS

Peptide Synthesis

The biotinylated peptide construct was synthesized and purified according to protocols previously described (5,6).

Cell Lines and Cell Culture

RIF-1 cells were obtained from Dr. Brian Wilson (Princess Margaret Hospital/Ontario Cancer Institute, Toronto, Canada) and grown as monolayers in α -minimal essential medium (α -MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) (ImmunoCorp., Montreal, Canada), penicillin (10 mL), streptomycin (100 μ g/mL) and 1 mM glutamine at 37°C with 5% CO₂. Growth medium was changed every 2–3 days, and cells were subcultured twice weekly. Cells were counted using a hemocytometer.

Preparation of Cell Samples for Electron Microscopy

RIF-1 cells were dispensed (10^6 cells/mL) into medium containing a biotinylated construct at a final concentration of $0.01 \mu\text{M}$. Cells were incubated at 37°C without light, washed ($3\times$) with phosphate-buffered saline (PBS) then fixed for 6 h at room temperature in 4% paraformaldehyde/0.7% glutaraldehyde (0.1 M PBS, pH 7.2). Fixed cells were dehydrated through a series of alcohol solutions (50%–95% ethanol) then embedded in medium-grade LR White resin (Marivac, Halifax, NS). Sections (90 nm) of embedded cells were collected on 300 mesh nickel grids and labeled. Nonspecific binding was blocked with 1% (wt/vol) bovine serum albumin (BSA) in PBS for 5 min, before a 2-h incubation at room temperature in 25- μL drops of 15 nm colloidal gold-streptavidin conjugate ($21.7 \mu\text{g/mL}$; British Biocell International, Cardiff, England, UK) diluted 1:15 in sterile, deionized distilled water. Following two washes in 1% (wt/vol)

BSA in PBS and sterile, distilled water for 10 min, sections were post-fixed in 1% glutaraldehyde (diluted in water) for 5 min and rinsed $3\times$ in distilled water for 5 min. To enhance contrast, sections were counterstained with 5% uranyl acetate (in water) for 9 s before being examined and photographed in an Hitachi H600 Transmission Electron Microscope (Hitachi Scientific Instruments, Mountain View, CA, USA).

Quantification of Gold Particle-Labeling in Cells

The quantification of gold particles was conducted using electron micrograph images scanned (ARCUS II scanner; AGFA Graphics, Ridgefield Park, NJ, USA) into a Power Macintosh® 8500/150 computer (Apple Computer, Cupertino, CA, USA) and analyzed using Adobe® Photoshop® (Version 3.0; Adobe Systems, San Jose, CA, USA) and the public domain NIH Image program (Version 1.61; developed at the National Institutes of Health; <http://rsb.info.nih.gov/nih-image/>). Results

Figure 1. (a) Shows the tools available within the NIH Image *Tool Palette* window. (b) Shows the LUT window, which can be used to modify image contrast and color.

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were transferred from NIH Image into Cricket Graph (Version 1.3.2; Computer Associates International) for quantification.

Black and white images of photographic negatives were acquired (at a resolution yielding 600 dpi) through a plug-in (Fotolook, PS 2.09.2; AGFA Graphics) into an Adobe Photoshop file (color mode; RGB) with a transparent background. Images were edited by "cropping" unwanted regions and then converted to positive contrast images using the **Invert** command (under the **Image** icon). Gold particles are now seen as black dots. A scale bar was introduced onto the images to allow the spatial calibration of subsequent area measurements, and images were saved as tag image file format (TIFF) files. A specific region of the cell, such as the nucleus or, as in this case, the boxed area (Figure 2a), was selected using the *Lasso* tool in the Adobe Photoshop toolbox. This area was subsequently cropped out and saved in a separate TIFF file for further analysis. Manual tracings of subcellular regions were conducted using the keyboard mouse.

Background noise associated with uranyl acetate staining was removed using a number of Photoshop tools such as the *Eraser* function, *Brightness/Contrast* correction, the **Magic Wand** icon and the *Similar* function. The *Eraser* function in the toolbox was used to remove gold particles located outside the area-of-interest and the *Brightness/Contrast* function (under the **Image** icon) was used to "fade out" the contrast associated with uranyl acetate staining. Background noise was excluded using the **Magic Wand** icon. Clicking on a single gold particle (black dot) and then on the *Similar* function (under the **Select** icon), allows the *Magic Wand* tool to select pixels within a specific intensity range. The lower the tolerance level, the narrower the range of intensity included in the selection. A tolerance of ≤ 5 was found to be optimal for selecting all particles. Selected gold particles were copied and saved into a separate, new TIFF file, which was subsequently accessed through NIH image.

To ensure that all gold particles within the boxed area were counted, it was necessary to equilibrate the con-

trast of their representative pixels. The contrast of all selected pixels was made to fall within a narrow range of gray value (defined with both an upper and a lower limit by the threshold) by clicking into the **Options** icon and applying the *Threshold* function to drag the **Look-Up Table** (LUT; Figure 1a) icon up the LUT window (Figure 1b). The relative contrast of pixels is expressed as mean density (see Table 1). Individual gold particles were selected using the automatic outlining tool (Figure 1a). Commands for analysis are found by clicking on the **Analyze** icon. Counting was initiated using the **Analyze Particles** command, with each

particle individually numbered, and the results of the analysis were viewed using the *Show Results* function.

Particles were frequently clustered and counted as single objects, depending on the level of contrast and threshold applied (Figure 1b). To calculate the total number of particles within clusters, it was necessary to determine the area of each cluster. Using the straight line tool (select lines; Figure 1a), a scale bar of a known distance was introduced, and the image was spatially calibrated to that measurement. For this task, a line was drawn across the diameter of a single gold particle (15-nm diameter), and this value was entered

Figure 2. (a) Adobe Photoshop image of an electron micrograph depicting the 2-D cross-section through a RIF-1 cell treated with a biotinylated peptide construct subsequently revealed using gold particle-labeled streptavidin. The area selected for analysis is outlined by a box. (b) An enlarged image of the boxed area before removing background noise shows single and clustered gold particles as black dots. (c) Quantitative analysis of gold particles was conducted using NIH Image. Clustered particles were counted as single objects. Cluster size was calculated by dividing a cluster area by the average area for a single particle.

Table 1. The Results of Gold Particle Quantification

Object Number	^b Pixel Area	Mean Density	^a Cluster Size	^c Running Sum
1	670.31	221.79	4	4
4	535.32	222.29	3	12
10	321.76	221.81	2	29
13	615.77	221.88	4	35
14	715.93	222.19	4	39
21	40.82	219.73	0	49
32	196.12	222.61	1	83
34	687.05	222.41	4	88
37	323.60	222.04	2	93
49	859.53	221.98	5	127
58	869.93	222.33	5	143
62	498.13	222.50	3	151
69	369.44	222.41	2	166
77	367.65	222.30	2	185
82	181.05	221.16	1	194

^aCluster size represents the ^bpixel area of the object in nanometers (nm²) divided by the calculated average area for a single gold particle (166 nm²).

^cRunning sum represents the accumulated total number of gold particles for all objects shown in Figure 2C. Thresholds of gray values ensured that the mean density contrast was similar for all particles.

by the *Set Scale* function (to ensure that all clusters and single particles were included, the range of pixel size was set between 5 and 20 000). The area of each cluster or single particle was computed using the *Measure* function and viewed by the **Show Results** command. For mathematical analysis, results were copied into a Cricket Graph data file, and the total number of particles were calculated by dividing the area of clustered particles by the area of a single particle. The total number of gold particles was subsequently calculated using the *Running Sum* function found under the **Transform** icon.

Adobe Photoshop images were used to express the total number of particles per square micron (μm^2) area. Images were accessed within NIH Image, and the straight line tool was again used to *Set Scale*, this time using the scale bar on the image. The freehand tool (select lines; Figure 1a) was then used to out-

line the region-of-interest (whether it be the nucleus of the cell, or the whole cell or, in this case, the boxed area), and the resulting area was computed under the **Show Results** icon. It was also possible to determine the number of gold particles within the cytoplasm of a cell by subtracting the number of particles within the nucleus from the total number within the cell.

RESULTS AND DISCUSSION

The endocytic uptake and accumulation of a biotinylated peptide construct into RIF-1 cells provided a model to assess the potential for quantifying intracellular gold particles from electron micrographs using existing software packages. This study demonstrates that the combination of three commercially available software programs permits a fast, efficient and quantitative analysis

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of electron micrographs, allowing every discernible gold particle or cluster of gold particles within the 2-D area of a cell profile to be counted.

A small region of the cross-sectional area of a RIF-1 cell was analyzed for this report to ensure clarity and proof-of-concept. A high-resolution image (600 dpi) of an electron micrograph was acquired into Adobe Photoshop (Figure 2a), and a region was selected (outlined by the box) within one of the cytoplasmic vesicles, in which gold particles (black dots) were concentrated. Quantification of gold particle accumulation was conducted using the NIH Image program, which is one of the few 2-D image-processing packages compatible with Apple Macintosh computers and suited to electron microscopy (EM) (4). NIH image is compatible with a number of Macintosh file formats including TIFF, QuickDraw™ picture (PICT) and Photoshop. This program can measure

the area of a cell or the area of subcellular regions within a cell, together with the number of particles within each of those areas. Enlargement of the boxed area (Figure 2b; total magnification = $\times 23\,000$) revealed that many of the particles were tightly clustered, often with no obvious demarcation. *Thresholding* was used to eliminate background noise, ensuring that only gold particles were analyzed. The adequacy of thresholding was evident by the small difference in gray value between particles; mean density contrast = 221.84 ± 0.56 (Table 1). Further image enhancement utilizing *Thresholding*, *Sharpening* and *Contrast* functions helped to further optimize particle separation. Ultimately, the reproducibility of results of image analysis will be governed not so much by the protocol but by the consistency of sample preparation, including the level of uranyl acetate staining and the concentration of gold particle labeling.

To address this point, we have conducted a further experiment involving images in which the background staining was noticeably greater than that presented in Figure 2a. It was found that the ability to “fade out” the background noise using the *Brightness/Contrast Function* of Photoshop was just as effective in these more densely stained electron micrographs (results not shown). However, the constraints associated with the resolution of the image meant that the total number of individual gold particles within clusters was often not deciphered, such that clustered particles were counted as single objects. Consequently, a total of 83 individual objects were counted within the boxed area (Figure 2c). Fortunately, the inability to segment clustered particles does not limit the accuracy of this method since the numerical analysis provides a measure of particles per area rather than strategy to identify individual particles.

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The total number of particles within a cluster (cluster size) was determined by the area of the cluster. The known diameter of a single particle (15 nm) was used for spatial calibration from which the size and resolution of the area was estimated to be $3.44 \mu\text{m}^2$ and $10.5 \text{\AA}$, respectively. The pixel area of a single gold particle (98.34 pixel squares; 0.746 pixels/nm) was converted to square microns (μm^2) using the *Set Scale* function in NIH Image to allow a direct comparison with other electron micrograph images. The average area of a single particle ($0.166 \mu\text{m}^2$) was used instead of the known area ($0.176 \mu\text{m}^2$) to eliminate any change in size as a result of image distortion. However, the difference in area ($0.010 \mu\text{m}^2$) suggests that distortion is not a major consideration.

Arithmetic functions within Cricket Graph were used to calculate cluster size by dividing the area of the cluster (calculated using the spatial calibration) by the average area of a single particle ($0.166 \mu\text{m}^2$). The number of single and clustered particles was tabulated, and the total number of particles within the selected area was calculated to be 195. The size of the analyzed area ($3.44 \mu\text{m}^2$) was then used to determine particle density: 56.7 particles per μm^2 .

The results of 15 randomly chosen objects out of the 83 (Figure 2b) are shown in Table 1. A comparison between the calculated values of cluster size shown in Table 1 and the actual cluster size determined by manually counting the particles shown in Figure 2b confirms the accuracy of this technique. For example, the calculated cluster size of object "65" is six, and six particles of gold can clearly be seen in Figure 2b. Results were consistent for all analyzed objects. Furthermore, particles, such as "21", that were bisected by the boundaries of the selected box, can be excluded from the analysis to allow accurate data acquisition using the *Ignore Touching Edge* function in the **Particle Analysis Options** window in NIH Image. Alternatively, the boundaries of the area to be analyzed can also be established by the experimentalist as a non-rectangular outline using the freehand *Lasso* tool in Photoshop. In this way, the boundaries could be set to avoid bisection of gold particles. In this instance, however, the bisection of par-

ticle 21 resulted in its area value being rounded to zero and was therefore not included in the count.

Computer image processing in biological EM has progressed considerably since initial reports of its use over thirty years ago (3). Unfortunately, many current electron microscope image-analysis packages are designed for use on workstations and PCs (2) and are better suited to more experienced computer users. The increasing use of Apple Macintosh computers in research and clinical laboratories has prompted the development of commercially available Macintosh-based, image-processing software such as Photoshop. Photoshop remains one of the fore-runners for 2-D image manipulation, and the ease of optimizing image quality using this software package can be fully exploited when used in conjunction with NIH Image for maximizing data acquisition. The versatility of our approach is further enhanced by the fact that both Adobe Photoshop and NIH Image software versions are available for PC-based systems (for further details see the Adobe Photoshop Web site: <http://www.adobe.com/proindex/photoshop>, the NIH Image home page Web site: <http://rsb.info.nih.gov/NIH-image/Default.html> or the Scion Corporation home page Web site: <http://www.scioncorp.com/>). Furthermore, these programs can also be used in combination with Macintosh-based stereological analysis systems, offering additional quantitation features including color-coded categorizing of data (1). The ability to group sets of data and grid-plot images is also offered by Image Pro[®] software (Versions 3.0/4.0; Media Cybernetics, Silver Spring, MD, USA; see Image-Pro Plus Web site: <http://www.mediacybernetics.com/>). This software, as in the case of Adobe Photoshop, is also available in both Macintosh and PC formats. Image-analysis software, like AnalySIS[®], allows the electron image produced within the microscope to be captured directly onto a computer (for details see the Soft Imaging System GmbH home page Web site: <http://www.soft-imaging-web.de/>). The convenience of this system, however, must be weighted against the relative increase in cost associated with

the installation and purchase of peripheral equipment, and the technical expertise required to maintain and operate both hardware and software components of this system. This study shows that accurate estimation of gold particle labeling can be achieved using a combination of commercially available software, allowing the rapid acquisition and elegant presentation of data.

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