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Investigating autophagy:

Quantitative morphometric analysis using electron microscopy

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Abstract

Autophagy is a compensatory pathway involving isolation and subsequent degradation of cytosolic material and organelles in eukaryotic cells.[1] The autophagic process can provide a "housekeeping" function by removing damaged proteins and organelles in a selective or nonselective fashion in order to exert a protective effect following stress.[2] Remarkably, after being discovered to be much more of a targeted process than a random one, the role of autophagy became implicated in many normal cellular and disease processes.[3] Several methodologies are routinely employed to monitor the entire autophagic process.[4] Microtubule-associated protein light chain 3, a mammalian homolog of yeast Atg8, has been widely used as a specific marker to monitor autophagy in numerous cell types.[5] While monitoring autophagic flux is extremely important, it is also beneficial to perform a detailed analysis by electron microscopy (EM) to evaluate changes in various autophagic structures, quantify the areas involved, and determine if any particular organelle(s) or area of the cell cytoplasm is being targeted for degradation.[6] The following article describes methods to localize and quantify subcellular areas of autophagy using transmission EM. Also discussed are methods for subcellular localization of specific proteins by employing immunogold EM; this method becomes particularly useful in detecting early changes in cellular homeostasis that may occur before later signs of cellular insult can be observed morphologically.

Keywords

autophagy; electron microscopy; immunogold; morphometry; ultra-structure

1. Introduction

Autophagy is a catabolic process involving specific steps, including formation of a limiting double membrane, isolation of cytoplasm and/or organelles within the membrane (autophagic vacuole), and fusion of the autophagic vacuole with a lysosome and subsequent degradation of sequestered material.[7] Autophagy was first discovered by electron microscopy in the 1960s and for years thereafter it was thought to be a rather simple yet mostly random cellular event. Only in the past few decades has it come to light that

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autophagy could not only be a targeted mechanism of protein degradation, it may also play a pivotal role in other cellular processes, including development and aging[2,3] and in diseases such as cardiomyopathy, neurodegeneration, and cancer.[3,8,9]

Decades of investigation have led to the development of numerous methodologies used for qualitative identification of autophagic events as well as quantitative determination of how effectively the whole autophagic degradation process is (or is not) functioning in eukaryotic cells.[4,10] In most areas of research, electron microscopy (EM) has been used in a strictly qualitative fashion for identification of ultrastructural morphology or to localize subcellular proteins using immunocytochemistry. While these methods can be invaluable in and of themselves, it is also important to determine whether there are significant quantitative changes in the observed pathologies. Increases in total cytoplasmic area occupied by autophagic vacuoles may signify upregulation in cellular defense mechanisms in response to a given stress (e.g., environmental, toxicologic, etc.,). Concurrently, an increase in total cytoplasmic area occupied by later stages of autophagy (e.g., autophagolysosomes and residual products such as lipofuscin) may signify a cell's failure to tolerate and overcome a particular stress.[6] Obtaining quantitative information by EM, whether from morphometry, immunogold labeling, or a combination of both, can provide invaluable insight into what events were taking place inside a cell as well as aid in the interpretation of molecular and biochemical data.

The following is an in-depth approach using EM methods that have been previously described.[6,11,12] While Oberley et al.[6] briefly describe methods for the utilization of EM in a quantitative analysis of autophagy in rat hepatocytes following heat stress, it is important to keep in mind that the following methods are not strictly limited to autophagy. The methods described in the following article can be used to quantitate almost any subcellular feature or to localize and/or quantitate a variety of proteins by immunogold. When performed correctly, quantitative EM analysis of cells and tissues can provide valuable information and aid in the interpretation of data gathered by other methods.

2. Materials

2.1. Specimen collection and fixation for routine EM

Tissue fixative (2.5% glutaraldehyde): for glutaraldehyde fixative, stock buffers must first be prepared in distilled water. Sorenson's stock buffer "A" is 67 mM dibasic sodium phosphate and Sorenson's stock buffer "B" is 67 mM monobasic potassium phosphate. Mix 144 ml of Sorenson's stock buffer "A", 56 ml of Sorenson's stock buffer "B", and 1 ampoule (10 ml) of 50% EM grade glutaraldehyde (unless otherwise noted, all reagents and supplies in the following methods are from Electron Microscopy Sciences, Hatfield, PA). The pH should be 7.2–7.4 without having to adjust. Both Sorenson's buffers and the 2.5% glutaraldehyde fixative are stable for up to 6 months at 4°C (see Note 1).

Sorenson's phosphate wash buffer: for 500 ml of wash buffer, mix 363 ml of Sorenson's stock buffer "A", 137 ml of Sorenson's stock buffer "B", and 5 g sucrose. Wash buffer is stable for up to 6 months at 4°C.

Tissue post-fixative (Caulfield's fixative): Caulfield's fixative is prepared under a certified fume hood in a series of three steps as follows (see Note 2):

[1]Factors affecting the quality of fixation are specimen size, osmolarity, buffers, fixative penetration, temperature and duration of fixation, and fixative concentration.[13] Because the mode of fixation is immersion, the delay between harvesting of tissues and immersion in fixative must be made as short as possible. Glutaraldehyde is a cross-linking agent that stabilizes proteins and reacts with amino groups; however, its reaction with lipids, polysaccharides and mucosubstances is slight. To be most effective, glutaraldehyde should not be used to fix tissues greater than 1 mm^3 in size.

a. Make 2% osmium tetroxide (OsO_4) by adding 4 g of EM grade osmium tetroxide to 200 ml distilled water in a dark bottle with a screwtop lid. Close the bottle, seal with paraffin film, place in a sealed plastic bag, and store inside a labeled Styrofoam container at 4°C. Allow OsO_4 to dissolve overnight. Two percent OsO_4 is good for 6 months at 4°C.

b. Make Caulfield's buffer stock solutions. For Caulfield's stock solution "A", add 1.47 g sodium barbital and 9.7 g sodium acetate trihydrate to 50 ml distilled water. For Caulfield's stock solution "B", add 4.3 ml concentrated hydrochloric acid to 500 ml distilled water. Both stock solutions are good for 6 months at 4°C.

c. To make Caulfield's fixative (sucrose balanced osmium fixative): Mix together 20 ml Caulfield's stock solution "A", 20 ml Caulfield's stock solution "B", 50 ml 2% OsO_4, and 10 ml distilled water. Caulfield's fixative should be tightly sealed, wrapped in paraffin film, placed in a sealed plastic bag, and stored in a Styrofoam container at 4°C for up to 6 months. Just prior to use, add 0.045 g/ml of sucrose to the appropriate amount of Caulfield's fixative required for a particular experiment.

Dehydration reagents: graded ethanols should be prepared with 100% ACS grade ethanol and distilled water (e.g., 50%, 75%, 95% ethanol). EM grade propylene oxide is highly flammable and must be stored properly.

EMbed-812 resin mixture: for 100 ml of resin, mix together 46 ml of EMbed-812 resin, 28 ml of DDSA (dodecenyl succinic anhydride), 24.5 ml NMA (methyl-5-norbornene-2,3-dicarboxylic anhydride), and 1.5 ml of DMP-30 (2,4,6-tri(dimethylaminomethyl) phenol). Resin can be stored at −20°C for 3 months (see Note 3). Warm to room temperature before use.

2.2. Specimen collection and fixation for immunogold EM

Carson Millonig's fixative: add 1.86 g sodium phosphate monobasic and 0.42 g sodium hydroxide to 90 ml distilled water. Add 10 ml 37–40% formaldehyde solution. pH fixative to 7.2–7.4 with 1 N hydrochloric acid. Fixative should be made fresh before each use.

Dehydration agents: graded ethanols should be prepared with 100% ACS grade ethanol and distilled water (e.g., 60%, 80%, 95% ethanol). LR White resin is used 2:1 with 95% ethanol for dehydration/infiltration and neat for both infiltration and embedding in size 3 gelatin capsules.

2.3. Sectioning, staining and imaging of specimens

0.5% Toluidine blue stain: to make 0.5% toluidine blue, used for staining thick sections, mix together 19 ml of 0.2 M sodium phosphate monobasic, 81 ml of 0.2 M sodium phosphate dibasic, and 0.5 g of toluidine blue O. Store stain at room temperature for up to 6 months after preparation. Filter before use.

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[2]Glutaraldehyde has no "staining" properties to aid in visualization of subcellular tissue morphology, therefore osmium tetroxide is used because it combines fixation with the ability to give good contrast to tissues by EM.[13] Fixation by osmium tetroxide occurs by oxidation of unsaturated bonds of lipids and its differential binding results in the important delineation of phospholipid membranes of the cytoplasm.

[3]If no stored resin is available, it is best to make the EMbed-812 resin mixture the day before it will be needed in order to verify quality. Fill a plastic size 00 BEEM™ capsule with the resin mixture and place in a 65°C oven overnight to make sure the resin polymerizes properly. For convenience, store mixtures of EMbed-812 resin in 30 ml syringes capped and sealed with paraffin film. It is significant to point out that there are other epoxy resins in addition to EMbed-812 that are available for embedding, such as Araldite and Spurr's.[13] While the above protocol has been optimized for use with EMbed-812 resin, the adjoining clinical electron microscopy lab uses Spurr's as it has a lower viscosity and can be used for rapidly processing specimens in a microwave; however, this laboratory has found that Spurr's does not offer the same high level of specimen contrast as the EMbed-812 resin.

7.7% uranyl acetate (UA): mix 7.7 g of ACS grade uranyl acetate ($UO_2(OCOCH_3)_2 \cdot 2H_2O$) in 100 ml of distilled water in a dark bottle to make a saturated aqueous solution (see Note 4). Filter before each use and discard if precipitate starts to form. Store UA at room temperature for up to 3 months after preparation.

Reynolds lead citrate (for routine EM samples only): mix 1.33 g of ACS grade lead nitrate ($Pb(NO_3)_2$) (see Note 5), 1.76 g ACS grade sodium citrate ($Na_3(C_6H_5O_7)$), and 30 ml carbon dioxide-free distilled water in a glass-stoppered 50 ml volumetric flask (see Note 6). Shake vigorously for 30 min to complete the conversion of lead nitrate to lead citrate (will become a milky white solution). Once converted, add 8 ml of 1 N sodium hydroxide (1 g sodium hydroxide in 25 ml carbon dioxide-free distilled water) and mix until solution clears completely. Add carbon dioxide-free distilled water to the volumetric flask to bring the solution to a final volume of 50 ml. Store at room temperature in a tightly sealed plastic bottle for up to 3 months following preparation. Discard solution if precipitate starts to form.

3. Materials

Glass knife strips (6.4 mm × 25 mm × 400 mm) cut with a glass knife maker for use in cutting thick sections (1 μm sections) on an ultramicrotome. Thick sections should be mounted on charged slides [e.g., Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA)] to ensure tissue adhesion. Ultrathin sections (70–80 nm) should be cut on water using a diamond knife and tissue sections mounted on either square 300 mesh thin bar copper grids (routine EM) or square 300 mesh nickel grids (immunogold EM). Electron micrographs should be recorded either with a digital electron microscope or on Kodak EM 4489 negative film. If using film, images need to be digitized with a high resolution negative scanner (e.g., Epson Perfection 4870).

Immunogold labeling of specimens

Tris buffered saline (TBS) rinse: for 100 ml of 0.05 M TBS rinse buffer, make a 1:10 dilution of Tris stock buffer (0.5 M Tris, pH 7.2–7.4) in distilled water and add 0.9 g sodium chloride. Buffer can be stored at 4°C for 6 months.

Phosphate buffered saline (PBS) rinse: for 1 L of PBS rinse (pH 7.4), add 0.39 g sodium phosphate monobasic, 1.07 g sodium phosphate dibasic, and 8.5 g sodium chloride to 1 L of distilled water. Buffer can be stored at 4°C for 6 months.

Acetylated bovine serum albumin (BSA-c) block: for 10 ml of BSA-c block, make a 1:20 dilution of Aurion BSA-c™ in PBS. Block can be stored at 4°C for 1 month.

Antibody diluent: antibodies may be diluted in either TBS rinse buffer, PBS, or a buffer from a commercial source. For the protocol detailed below, a commercially available phosphate buffered Normal Antibody Diluent (ScyTek Laboratories, Logan, UT) was used.

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[4]Uranyl acetate is used to increase contrast in specimens and allow for increased resolution of images; however, uranyl acetate dissolves slowly in water. After adding the uranyl acetate to distilled water in a brown glass bottle, seal the bottle and sonicate for 20–30 min to dissolve. Uranyl acetate is a highly toxic and radioactive material. Avoid breathing dust and contact with skin or eyes. Dispose of aqueous uranyl acetate according to local, state and federal regulations.

[5]Reynolds lead citrate solution is used in conjunction with uranyl acetate to further enhance contrast and image resolution. Lead nitrate is extremely toxic and may be fatal if swallowed. Precaution should be taken to prevent inhalation of dust.

[6]Lead citrate will form precipitates when mixed with carbon dioxide (CO_2) in either water or room air. To make CO_2-free distilled water, fill 125 ml glass screw-top bottles with 100 ml double distilled water. Place caps on bottle until tight and then unscrew half a turn to loosen. Either boil the bottles in a water bath for 30 minutes or place autoclave tape on the cap and autoclave on the liquid cycle for 30 minutes. When removing bottles from the boiling water or autoclave, firmly tighten the screwtops immediately. When making Reynolds lead citrate solution, the CO_2-free water bottles should “hiss” when opened; if they don’t, use a different bottle of water. Because not all bottles of water may become completely CO_2-free, it is best to prepare several bottles at one time to ensure you will have at least one that will work to make the solution.

TBS wash buffer: for 100 ml of TBS wash buffer, make a 1:10 dilution of BSA-c block solution with 0.05 M TBS rinse. Buffer can be stored at 4°C for 6 months.

Alkaline Tris wash buffer: alkaline Tris wash buffer is a 1:10 dilution of 1 M alkaline Tris stock (pH 8.2) in distilled water. Buffer can be stored at 4°C for 6 months.

Antibodies and serums: primary antibodies of interest as well as normal serums to be used as negative controls can be purchased commercially. Fifteen nm gold-conjugated secondary antibodies (B.B. International, Cardiff, UK) are diluted to either 1:75 [anti-rabbit IgG (H + L)] or 1:50 [anti-mouse IgG (H + L)] in normal phosphate buffered antibody diluent.

4. Methods

4.1. Specimen collection and fixation for routine EM

Perform all EM work in a fume hood as many of the chemicals and reagents are extremely toxic. Tissues must be collected immediately upon euthanization and immersed in the appropriate fixative as soon as possible (see Note 7). Dissect out a small piece of tissue (~3 mm^3) and place it in a shallow glass dish with 2.5% glutaraldehyde fixative. While immersed in fixative, further dissect the piece of tissue into 1 mm cubes, transfer to a labeled screwtop glass vial filled with fixative (the fixative volume should be roughly 20 times greater than the tissue volume), and fix overnight at 4°C. Decant fixative and rinse tissues 2 × 5 minutes in Sorenson's phosphate wash buffer.

Prepare for post-fixation in osmium tetroxide (OsO_4) by adding 5 ml of Caulfield's fixative to a vial containing 0.225 g sucrose. When the sucrose has dissolved, decant the Sorenson's wash buffer and replace with an appropriate amount of sucrose-balanced OsO_4. Cap the vial tightly and place at 4°C for 1 hour (the OsO_4 will turn the tissues black as it reacts with the unsaturated bonds of lipids). Discard OsO_4 into a proper container for disposal (see Note 8). Rinse tissues 2 × 5 minutes in Sorenson's phosphate wash buffer.

Dehydrate tissues in a graded series of ethanols for 10 minutes each: 50%, 75%, 95%, 2 × 100%. After the first addition of 100% ethanol, make sure to cap the vial to prevent moisture from entering the vial. Further dehydrate tissues 2 × 10 minutes in propylene oxide.

Mix propylene oxide 1:1 with the EMbed 812 resin mixture and add to the tissues. Infiltrate for 2 hours while on a rotary shaker. Decant used EMbed 812 resins into a 250 ml plastic tri-pour beaker (see Note 9).

Add pure EMbed 812 resin mixture to the tissues and infiltrate for 1 hour while on a rotary shaker.

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[7]Perfusion fixation is ideal for analysis by EM; however, in many studies the tissues will also be used for other molecular and biochemical analyses that require fresh tissues so perfusion fixation is not possible in these instances. For collection of tissues for immunogold studies detailed in section *3.2*, it is important to point out that other techniques such as cryoultramicrotomy and pre-embedding labeling of specimens may be employed.[13] There are several factors to consider first, however, as both cryoultramicrotomy and pre-embedding can cause a significant decrease in the quality of tissue morphology. Cryoultramicrotomy also requires rather large investments in both in time and money, involves a great deal of technical skill, and tissues cannot be stored for long periods of time for use in future studies. Pre-embedding studies have been successful when there is little to block access of the label to the antigen and therefore has been most effective in detection of cell surface membrane antigens.

[8]OsO_4 must be properly neutralized before it can be disposed. Perform all work in a certified fume hood. Fill a 250 ml plastic tri-pour beaker with 125 ml of corn oil (corn oil is used because of its numerous unsaturated lipid bonds). Discard the OsO_4 into the beaker with corn oil, taking care not to fill the beaker to more than 200 ml. To confirm that neutralization of the OsO_4 has occurred, place a large piece of filter paper soaked in corn oil over the top of the beaker and let it sit for 24 hours. If the filter paper does not turn black then neutralization has occurred. The mixture is now safe for disposal following all local, state, and federal guidelines.

Prepare size 00 BEEM™ capsules with a paper label containing an accession number that is unique to each specimen for identification purposes. Add EMbed 812 resin mixture to the BEEM™ capsules, making sure to remove any air bubbles. Place one piece of tissue into the conical bottom of each BEEM™ capsule and fill the capsule to the top with the EMbed 812 resin mixture.

Place the capsules into a 65°C oven overnight to polymerize.

4.2. Specimen collection and fixation for immunogold EM

Perform all EM work in a fume hood as many of the chemicals and reagents are extremely toxic. Tissues must be collected immediately upon euthanization and placed in the appropriate fixative as soon as possible (see Note 7). Dissect out a small piece of tissue (~3 mm^3) and place it in a shallow glass dish with Carson Millonig's fixative. While immersed in fixative, further dissect the piece of tissue into 1 mm cubes, transfer to a labeled screwtop glass vial filled with fixative (the fixative volume should be roughly 20 times greater than the tissue volume), and fix for 1–2 hours at room temperature. Decant fixative and rinse tissues 2 × 5 minutes in Sorenson's phosphate wash buffer.

Dehydrate tissues in a graded series of ethanols: 2 × 10 minutes in 60% ethanol, 2 × 10 minutes in 80% ethanol, and 2 × 30 minutes in 95% ethanol. After the first addition of 95% ethanol, make sure to cap the vial to prevent excess moisture from entering the vial.

Mix LR White resin 2:1 with 95% ethanol and add to the tissues. Infiltrate for 1 hour while on a rotary shaker. Decant waste resin mixture into a 50 ml tube with screw cap (see Note 9). Add pure LR White resin and infiltrate overnight at room temperature on a rotary shaker.

On day 2, decant LR White resin into a 50 ml tube and add fresh LR White. Infiltrate for 1 hour at room temperature on a rotary shaker.

Prepare size 3 gelatin capsules with a paper label containing an accession number that is unique to each specimen for identification purposes. Add LR White resin to the bottom portion of the capsules, making sure to remove any air bubbles (see Note 10). Place one piece of tissue into the bottom of each gelatin capsule and fill the capsule to the top with LR White. Place the top half of the gelatin capsule over the bottom half and make sure it "locks" closed and check to ensure there is an airtight seal.

Place the capsules into a 55°C oven overnight to polymerize.

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[9]EMbed 812 resin mixtures and LR White resin must be polymerized before they can be disposed. For the EMbed 812 resin mixture, decant both the 1:1 propylene oxide:EMbed 812 resin mixture and pure EMbed 812 resin from the infiltration steps into a 250 ml plastic tri-pour beaker. For LR White resin, decant both the 2:1 LR White:95% ethanol mixture and pure LR White from the infiltration steps into a 50 ml tube with a screwtop cap (LR White does not polymerize in the presence of air). Allow the beaker or tube (without cap) to sit overnight in a certified fume hood to evaporate the propylene oxide or ethanol from the resin. Once the propylene oxide has evaporated, the plastic beaker can be placed in a 65°C oven to polymerize overnight. Once the ethanol has evaporated from the LR White, tightly seal the vial with the screw cap and place in a 55°C oven to polymerize overnight. When polymerized, both resins can be disposed following local, state and federal guidelines.

[10]The gelatin capsules must be sealed tightly after the tissue is added and every effort must be made to keep as much air as possible from being enclosed in the gelatin capsules. LR White does not polymerize when exposed to air so the less air that is trapped in the gelatin capsule, the better the LR White will polymerize. It is significant to point out that there are other hydrophilic resins in addition to LR White that are available for embedding, such as Unicryl and Lowicryl, JB-4, and methylacrylate.[13] The above protocol has been optimized for use with LR White resin as other resins have been shown to not be as effective with post-embedding immunogold protocols[26] or because resins such as Lowicryl are more suited to low temperature applications. Results from immunogold studies performed in the authors' laboratory on a variety of cells and tissues can be seen by referring to past publications.[6,11,14–17]

4.3. Sectioning, staining and imaging of specimens

For detailed methods on performing ultramicrotomy for obtaining both thick and thin sections, please consult a comprehensive EM text.[13]

After obtaining thick sections on a drop of water, place the glass slide on a hot plate set on "low" to dry. When the drop of water has evaporated, add enough 0.5% toluidine blue to cover the sections and place slide again on the hotplate. Stain EMbed 812 resin sections for ~1 minute but only stain LR White sections for ~20 seconds (LR White is hydrophilic and absorbs the stain more readily and can become too dark if the stain is left on too long).

Gently rinse the toluidine blue from the slides using distilled water. Dry the slide and observe the thick sections under a brightfield microscope. Choose a tissue block for thin sectioning that has the best morphology and available surface area for what is to be studied. Trim the chosen block down to 1–2 mm^2 if possible as this will fit well with the cutting face of the diamond knife.

After cutting thin sections, collect a 0.5 μm after-section on a slide, stain with 0.5% toluidine blue, and observe under a microscope to ensure the area of interest has not been lost or cut through. Pick up thin sections with a 300 square mesh grid (copper for EMbed 812 samples and nickel for LR White samples), blot the grids on filter paper to dry, and place in a suitable container for storage (see Note 11).

For tissues on nickel grids for immunogold analysis, proceed to section *4.4*. To stain specimens on copper grids for routine EM analysis, float the grids tissue side down on a drop of 7.7% uranyl acetate (UA) in a Teflon well plate. Place the Teflon plate in a 55°C oven for 30 minutes. Remove the plate from the oven, carefully rinse the grids with distilled water, and blot dry with filter paper.

Next, float the grids tissue-side down on a drop of Reynold's lead citrate in a Teflon well plate for 5 minutes at room temperature. Carefully rinse the grids with distilled water and blot dry with filter paper. The samples are now ready to be viewed under the electron microscope.

4.4. Immunogold labeling of specimens

Place 1–2 paper towels into the bottom of a round 15 cm dish and wet thoroughly with distilled water (see Note 12). Place 4 labeled 12-well Teflon slides (5 mm diameter) on top of the paper towels, one for each of the following incubations in TBS rinse, BSA-c, PBS and primary antibody.

Add one drop (~30 μl) of filtered TBS rinse to each well of the 1st slide (1 well per nickel grid), one drop of filtered BSA-c block to each well of the 2nd slide, one drop of filtered PBS rinse to each well of the 3rd slide, and one drop of primary antibody or normal serum prepared to the desired dilution to each well of the 4th slide (see Note 13).

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[11]Special attention must be paid to the antibodies employed for immunogold EM. If the antibody is against a protein used to detect oxidative or nitrative damage products, the grids must be placed under vacuum (usually in a vacuum desiccator) as soon as they have been collected. This prevents further oxidation of the tissues from room air which could possibly result in false positive (or in the case of some oxidative DNA damage markers, false negative) results.[27]

[12]The first four steps of immunogold labeling are performed using a humidified chamber which can be constructed simply by placing a wet paper towel in the bottom of a large plastic dish with a lid and placing the slides on top of the paper towel. When performing studies under vacuum, it is of tremendous importance to use a humidified chamber as to prevent evaporation of reagents during the overnight incubation in primary antibody at 4°C.

[13]All reagents (e.g., TBS rinse, BSA-c block, and PBS) are placed in separate 10 ml sterile Luer-Lok™ syringes with a 0.22 μm surfactant free cellulose acetate (SFCA) syringe filter. Once each filtered reagent has been added to the slide, remove the SFCA filter and wrap the syringe tip with paraffin film. Labeled syringes can be stored at 4°C for 1 month.

Using forceps, place each grid tissue-side down on a drop of TBS rinse. Incubate on TBS rinse for 10 minutes.

Using an inoculating loop or forceps, transfer grids to the next slide containing BSA-c. Incubate for 30 minutes.

Transfer grids to the next slide containing PBS rinse. Incubate for 5 minutes.

Transfer grids to the last slide containing the primary antibody or normal serum. Incubate grids overnight at 4°C inside the humidified chamber. If using antibodies which require the samples to be under vacuum while on the primary antibody overnight, place the humidified chamber inside of a vacuum desiccator and evacuate the chamber for approximately 5 minutes. Securely clamp the vacuum tubing, remove tubing from the vacuum source, and place the desiccator at 4°C overnight.

On day 2, warm the grids at room temperature for 1–2 hours. The following wash steps should be performed in a 96-well flat bottom sterile culture plate.

Float grids on 4 changes of filtered TBS wash buffer for 5 minutes each while on a rocker.

Float grids on 1 change of filtered alkaline Tris wash buffer for 10 minutes while on a rocker.

Add one drop (~30 μl) of gold-conjugated secondary antibody prepared to the desired dilution to each well of the slide. Float grids tissue-side down for 90 minutes at room temperature.

Float grids on 2 changes of filtered TBS wash buffer for 10 minutes each while on a rocker.

Float grids on 2 changes of filtered distilled water for 5 minutes each while on a rocker.

Remove grids from distilled water with forceps and blot dry with filter paper.

Float grids tissue side down on a drop of 7.7% uranyl acetate (UA) in a Teflon well plate for 10 minutes at room temperature. Carefully rinse the grids with distilled water and blot dry with filter paper. The samples are now ready to be viewed under the electron microscope. Several references from the authors' laboratory are available to observe the results of using the above immunogold protocol on many different tissues (i.e., liver, heart, kidney, prostate and cell culture) using a variety of antibodies.[6,11,14–17] There are also studies from other laboratories investigating autophagy which demonstrate successful use of specific autophagic protein markers and immunogold labeling techniques.[18–21]

4.5. Quantitative morphometric analysis of electron micrographs

Using a transmission electron microscope operated at 75 kV, start at any given edge of the grid and begin surveying the specimen one grid square at a time in an orderly fashion to make certain that systematic uniform random sampling can be achieved.[22–25] In an effort to provide data from only a few tissue sections that can be considered representative of the tissue as a whole, it is absolutely critical to take steps throughout the entire experimental process to ensure random sampling. This includes selecting specimens and assigning experimental and control groups at random, collecting and processing random cells or pieces of tissue from the organ(s) and/or tissue(s) of interest with assignment of a unique accession number for each specimen to make ultrastructural analysis in a blinded fashion possible, and taking photos at a pre-determined random interval (i.e., one photo every 1–5 grid squares). A satisfactory number of micrographs, all at the same magnification, must be taken to

ensure statistical significance can be achieved. Save micrographs in a digital format (see Note 14) and print out an 8 × 10 inch copy of the micrograph on plain paper for future reference in analyzing damaged areas.

Open the digital images with photo imaging software suitable for observing high resolution images at increased magnification [e.g., Adobe Photoshop Elements (Adobe Systems Inc., San Jose, CA)] and find the corresponding printout of the image. Use different colored markers to circle each damaged organelle or specific area of interest (e.g., autophagic vacuoles, autophagolysosomes, mitochondria, etc.) on the printout or to number the organelles/areas for immunogold (see Note 15).

Before opening images in the program used for quantitation [Scion Image Beta 4.02 (Scion Corporation, Frederick, MD)], it may be necessary to reduce the file size. The entire image should be viewable on the computer screen when opened in Scion Image; if it is not, re-size the image until the entire image can be viewed on the same screen.

Once an entire image is viewable while in Scion Image (Fig. 1A), it is necessary to set the scale. If calibrating an image captured digitally with an electron microscope that has a scale bar embedded in the image, use the line measure tool in Scion Image to click on the entire length of the given scale bar. Go to the “Set Scale” option in the Analyze menu and the box for “Measured Distance” should now have a number for “Pixels”. Select micrometers as the unit of measure, make sure the pixel aspect ratio is “1.0000”, and input the “Known Distance” as that from the image scale bar (e.g., 5 μm). If calibrating an image captured on film and later converted to a digital image, the “Known Distance” must first be calculated. Multiply the negative length in millimeters (length of the viewable area only, not including the edges) by 1000 and then divide the result by the actual magnification of the negative. For example, if the negative length is 74 mm and the magnification is 3000x, the “Known Distance” will be 24.67 (74 × 1000/3000). In Scion Image, use the line measure tool to measure the distance from top to bottom of the viewable area of the image (excluding the black borders). Follow the steps above to set the scale using the value calculated for the “Known Distance.”

Under “Options” in the Analyze menu select the measurement options “Area,” “Include Interior Holes,” and Wand Auto-Measure.” Then select “Show Results” from the Analyze menu to view measured values as they are obtained.

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[14]When working with digital images, it is important to remember that many kinds of image manipulation can be construed to be misrepresentative of the actual data and much care should be taken to avoid such circumstances.[28] If using a digital electron microscope, save images in TIFF format, making sure to add the appropriate scale bar to the image. If using an electron microscope that requires negative film, develop negatives according to standard protocols. Using a high resolution negative scanner, scan the images in 8-bit grayscale with the unsharp mask filter option selected and save images in TIFF format. When printing an 8 × 10 image on plain paper, a laser printer set to basic quality should suffice; this image will be used as a reference only to mark areas of damage, record the number of gold beads for immunogold, etc.

[15]High resolution software is required because it has been the author’s experience that the program used to quantitate sub-cellular areas (Scion Image Beta 4.02) does not have suitable resolution capabilities for definitive morphologic identification of some subcellular features; therefore, the paper printout of the image is needed for landmark purposes when operating in Scion Image. For immunogold analyses, the printout is also necessary to number the organelles/areas of interest to ensure that the area measured for each organelle/ area is precisely matched in a spreadsheet to the number of gold beads counted for that particular area. Before beginning any type of analysis, it is very important to clearly outline the criteria to be used to assess damage or determine if any subcellular component is either missing or present when it should not be. Whenever possible, consultation with a board-certified pathologist who has experience working with electron microscopic images is highly recommended. There are also several textbooks available which illustrate examples of normal and abnormal pathologies and may also be helpful.[29] It is also important that only one person be responsible for the actual analysis of subcellular morphology for all images and interpret micrographs in a blinded fashion; this type of analysis can be subjective and to one person a particular area or organelle may look as if it fits the established analysis criteria while another person may not assess it the same way. This would introduce too much variability and could falsely increase/ reduce the significance of the results.

Choose the paintbrush drawing tool, set the appropriate brush size (e.g., 2–4 pixels), and begin circling areas of interest as shown in Figure 1B. Make sure that no two areas touch one another or else the program will not be able to distinguish between the two areas and they will be measured together as one area by the imaging program.

Once all areas have been circled, select “Threshold” from the Options menu and drag the threshold bar in the “LUT” palette all the way to the bottom to obtain a blank white area with only the black circles visible (Fig. 1C).

Select the wand tool and click on the edge of each black circle. The resulting area should appear in the “Show Results” window. When all measurements have been obtained, copy and paste the values into a spreadsheet for analysis.

Several types of data analysis can be helpful in determination of subcellular morphology changes (i.e., changes resulting in/from autophagy, changes in/from oxidative/nitrative damage, etc.,). These include calculating the percentages of a particular organelle that may be damaged, the percentage of cellular cytoplasm occupied by the nucleus, myelin figures, lipid or autophagic events, and the changes in the average density (# gold beads/relative μm^2 area) of a particular protein of interest.[6,11]

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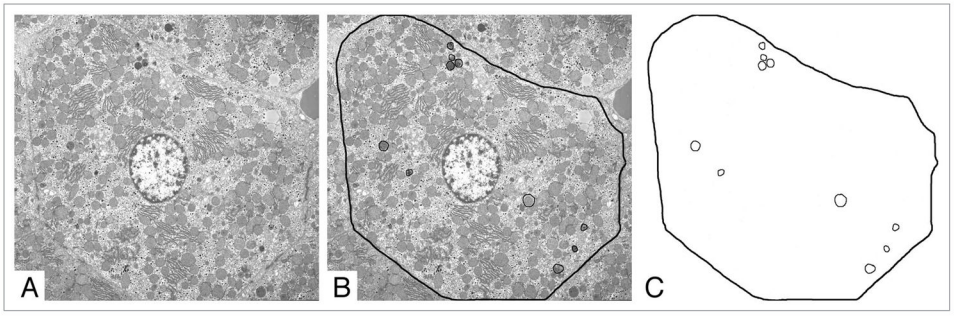

Figure 1.

Quantitative morphometric analysis of an electron micrograph. (A) representative micrograph of a rat hepatocyte photographed at low magnification to ensure the entire cell fit into the field of view and could be analyzed. (B) The borders of the hepatocyte are traced in the image analy -sis program, as well as other specific areas of interest for quantitation of area. In this example, areas of autophagic events autophagolysosomes, and lipofuscin) have been outlined. (c) After all areas have been circled, the image threshold is reduced to produce a pure black and white image. The wand auto-measure tool in the image analysis program can now be used to click on each individual circle to obtain area (μm^2) measurements.