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Quantifying Size and Number of Adipocytes in Adipose Tissue

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Abstract

White adipose tissue (WAT) is a dynamic and modifiable tissue that develops late during gestation in humans and through early postnatal development in rodents. WAT is unique in that it can account for as little as 3% of total body weight in elite athletes or as much as 70% in the morbidly obese. With the development of obesity, WAT undergoes a process of tissue remodeling in which adipocytes increase in both number (hyperplasia) and size (hypertrophy). Metabolic derangements associated with obesity, including type 2 diabetes, occur when WAT growth through hyperplasia and hypertrophy cannot keep pace with the energy storage needs associated with chronic energy excess. Accordingly, hypertrophic adipocytes become overburdened with lipids, resulting in changes in the secreted hormonal milieu. Lipids that cannot be stored in the engorged adipocytes become ectopically deposited in organs such as the liver, muscle, and pancreas. WAT remodeling therefore coincides with obesity and secondary metabolic diseases. Obesity, however, is not unique in causing WAT remodeling: changes in adiposity also occur with aging, calorie restriction, cancers, and diseases such as HIV infection. In this chapter, we describe a semiautomated method of quantitatively analyzing the histomorphometry of WAT using common laboratory equipment. With this technique, the frequency distribution of adipocyte sizes across the tissue depot and the number of total adipocytes per depot can be estimated by counting as few as 100 adipocytes per animal. In doing so, the method described herein is a useful tool for accurately quantifying WAT development, growth, and remodeling.

1. INTRODUCTION

White adipose tissue (WAT) is a dynamic and modifiable component of overall body mass in adulthood, comprising between ~3% and ~70% of total body weight (Hausman, DiGirolamo, Bartness, Hausman, & Martin, 2001). WAT develops in late (14–24 weeks) gestation in humans (Ailhaud, Grimaldi, & Negrel, 1992; Poissonnet, Burdi, & Garn, 1984) and postnatally in mice and rats (Han et al., 2011; Pouteau et al., 2008) and is unique in its potential for continuous and seemingly limitless growth, as observed in humans and animals under states of persistent energy surplus.

With obesity, the morphology and function of both individual adipocytes and whole WAT depots become altered. This process is a form of WAT remodeling. In periods of chronic positive energy imbalance (Poretsky & Ebooks Corporation, 2010), adipocytes store surplus energy as triacylglycerols, expanding in size (hypertrophy) and in number (hyperplasia) as a consequence. Adipocyte hypertrophy results from a relative increase in lipid deposition versus lipolysis (Karttinen, LaNoue, Martin, Vikman, & Ohisalo, 1995; Reynisdottir, Ellerfeldt, Wahrenberg, Lithell, & Arner, 1994). When demand for lipid storage exceeds the capacity of existing adipocytes, the pools of adipocyte precursors (preadipocytes) compensate by dividing and differentiating into adipocytes; this process, called adipogenesis, results in adipocyte hyperplasia (Cawthorn, Scheller, & MacDougald, 2012a, 2012b; de Ferranti & Mozaffarian, 2008; Faust, Johnson, Stern, & Hirsch, 1978). Impaired adipogenesis is believed to contribute to the development of metabolic comorbidities including type 2 diabetes, because engorgement of adipocytes with excess lipids triggers pathological changes to the adipose tissue. These changes include altered adipokine (e.g., chemerin) secretion, and increased adipose tissue inflammation due to macrophage infiltration and activation. Additionally, lipids that cannot be stored in adipocytes become elevated in the circulation and deposited ectopically in the liver, muscle, and pancreas (Goralski & Sinal, 2007; Le Lay et al., 2001; Ozcan et al., 2004; Roman, Parlee, & Sinal, 2012; Suganami & Ogawa, 2010). Together these metabolic abnormalities trigger systemic insulin resistance and abnormal insulin production, the basic pathology of type 2 diabetes. Obesity, however, is not alone in causing changes to WAT. Numerous diseases, developmental stages, and genetic animal models coincide with or result in WAT remodeling. For instance hypogonadism and Cushing's syndrome result in elevated and modified adipose deposition, whereas HIV infection, cachexia, and certain parasitic infections are marked by adipose tissue wasting (Desruisseaux, Nagajothi, Trujillo, Tanowitz, & Scherer, 2007; Lee, Pramyothin, Karastergiou, & Fried, 2013; Santosa & Jensen, 2012; Tchkonja et al., 2010; Tisdale, 1997). In addition, genetic modifications in transgenic animals including FABP4-Wnt10b, LXR $\beta^{-/-}$, SFRP5 $^{-/-}$, Timp $^{-/-}$, and many others all cause marked changes in adipocyte size or number compared to controls (Gerin et al., 2005, 2009; Longo et al., 2004; Mori et al., 2012). Accordingly, quantifying the number and size of adipocytes in the development, deposition, or remodeling of WAT is essential in characterizing the phenotype of a given adipose tissue depot.

Several methods have been described for quantitative histomorphometry of WAT (Bjornheden et al., 2004; Bradshaw, Graves, Motamed, & Sage, 2003; Chen & Farese, 2002; Hirsch & Gallian, 1968; Lee, Chen, Wiesner, & Huang, 2004; Maroni, Haesemeyer, Wilson, & DiGirolamo, 1990; Okamoto et al., 2007). The basis for a number of these techniques is to disrupt the tissue with collagenase allowing for isolation of the individual adipocytes, which are subsequently stained and/or analyzed by hemocytometer or coulter counter (Bradshaw et al., 2003; Hirsch & Gallian, 1968; Maroni et al., 1990). A limitation with these methods is that collagenase digestion, mesh separation, and/or centrifugation of adipocytes (Bradshaw et al., 2003; Hirsch & Gallian, 1968; Maroni et al., 1990) may damage or exclude certain cells. In contrast, flow cytometry lacks the drawbacks of mesh separation and centrifugation, instead separating adipocytes based on adipocyte size as a reflection of internal lipid accumulation (Lee et al., 2004). Flow cytometry, however, requires specialty

training and highly expensive equipment. Yet another reported approach takes advantage of lipophilic fluorophores to mark cellular lipids after which adipocytes size and number can be quantified using confocal fluorescent microscopy. In addition to the expensive microscope, a drawback with this method is the need to count 1000 adipocytes for accuracy, which is a relatively slow and tedious process (Nishimura et al., 2007). Osmium tetroxide is another powerful tool to mark cellular lipid content and has been used in conjunction with microcomputed tomography to visualize marrow adipocytes (see Chapter 7); however, this chemical is extremely toxic and can cause adipocyte swelling (Hirsch & Gallian, 1968). Finally, other investigators have described automated methods for quantifying hematoxylin and eosin (H&E) stained adipocytes. Each method, however, has a distinctive limitation minimizing their utility. For the method of Chen and Farese (2002), the inability to control for adipocytes touching the edge of the image results in an underestimation of adipocyte sizes. While the method of Osman et al. (2013) requires analysis of 16 representative fields of view per whole-slide image, which is laborious. In contrast to these previous approaches, we describe herein a quick and easy semiautomated procedure for histomorphometric analysis of paraffin-embedded adipose tissue that uses common and relatively safe laboratory reagents, basic laboratory skills, and standard laboratory equipment.

2. TECHNICAL ASPECTS

2.1. Tissue preparation

2.1.1 Obtaining and preserving tissues—Both human and rodent WAT are similar in terms of gross anatomical regions (subcutaneous or visceral depots) and morphology (Bjorndal, Burri, Staalesen, Skorve, & Berge, 2011). In humans, subcutaneous adipose depots accumulate beneath the dermis of the skin in multiple locations including the thigh, abdominal wall, and buttocks. In contrast, visceral adipose depots are located within the thorax (mediastinic) and abdomen (omental, mesenteric, perirenal, retroperitoneal, and parametrial) (Pond, Mattacks, Calder, & Evans, 1993). Rodents likewise have specific accumulation of subcutaneous and visceral WAT including epididymal, perirenal, mesenteric, pericardial, and inguinal depositions (for anatomy of mouse WAT depots see Cinti, 2005).

It is well established that increased visceral adipose tissue is highly associated with obesity-related cardiovascular and metabolic abnormalities when compared to subcutaneous depots (Brochu et al., 2001; Fujimoto et al., 1999; Kloting et al., 2010; Lau et al., 2011; Lefebvre et al., 1998; Nicklas et al., 2004). This difference coincides with a divergence in the expression of genes associated with glucose intolerance (increased *Glut4*, decreased *insulin receptor* (Lefebvre et al., 1998)) and atherosclerosis (increased *CCL4*, *CCL5*, and *IL-1 β*) (Lau et al., 2011). Unexpectedly, we have also observed differences in gene expression across a WAT depot. For example, with increasing distance from the testis, expression of *sFRP1* mRNA decreases, whereas expression of adiponectin mRNA increases in epididymal WAT (Fig. 6.1). Given this heterogeneity in function and/or expression between and within adipose tissues, it is important prior to necropsy to carefully consider which adipose depots, and the locations therein, will be of interest. For help in selecting the appropriate adipose depots, consult the method of Casteilla, Penicaud, Cousin, and Calise (2008).

During dissections, a standard necropsy procedure should be maintained between animals to minimize any variance in tissue collection. A wet-tissue weight is taken immediately after removal using an analytical balance. Special care is taken to remove any nonadipose-associated tissues from the depot including glands and lymph nodes. Once weighed, 100–500 mg of the adipose tissue of interest is placed in a sealable tube and covered by a ratio of >10 mL of 10% formalin per gram of WAT (Sigma-Aldrich, Cat# HT501320). Covered tissue is placed at 4 °C for 72 h, then switched to 70% ethanol (equal volume as was used for formalin) for 48 h. While extended formalin storage can result in difficulty with certain antigen detection due to “overfixation” of (Webster, Miller, Dusold, & Ramos-Vara, 2009), this has little impact on the current method as buffered formalin will not cause variable tissue hardening (Fox, Johnson, Whiting, & Roller, 1985). Of greater concern is the degradation of the tissue as a result of under-fixation and fixative procedures that lead to variable tissue “shrinkage.” A standard procedure should therefore be strictly followed for WAT fixing and dehydration.

1. Identify adipose tissue depot of interest
2. Carefully dissect out adipose depot removing nonadipose-associated tissue (e.g., glands, lymph nodes)
3. Weigh depot
4. Cover 100–500 mg of tissue with >10-fold volume (mL) of 10% formalin to tissue (g) and let sit at 4 °C for a minimum of 72 h
5. Remove tissue and place in 70% ethanol for 48 h

2.1.2 Tissue embedding—Preserved tissues are then placed in labeled histology cassettes (e.g., Cat# CA95029-822, CA18000-174; VWR International, Radnor, PA, USA) and paraffin (e.g., Paraplast Tissue Embedding Medium, Cat# 1006, Sigma-Aldrich, St. Louis, MO, USA) processed (e.g., Leica ASP 300 Paraffin Tissue Processor, Leica Microbiosystem, Wetzlar, Germany). Below are the steps used for paraffin processing adipose tissue.

1. 1 h in 70% ethanol
2. 1 h in 80% ethanol
3. 1 h in 95% ethanol (×2)
4. 1 h in 100% ethanol (×2)
5. 1 h in xylene (×2)
6. 1 h in 60 °C paraffin (×3)

Once the sample is processed, the tissue is embedded into a paraffin block (e.g., Tissue-Tek Paraffin Tissue Embedder, Sakura Finetek, Torrance, CA, USA) and stored at 4 °C. Time and care should be taken in the embedding process to ensure the tissue is placed into the center of the block. This will help standardize each sample for sectioning.

Note: Allowing the tissues to remain in the 60 °C paraffin for long periods of time after processing should also be avoided to limit tissue damage.

2.1.3 Sectioning, deparaffinizing, and staining adipose tissue—Embedded tissue samples are faced off using a low profile microtome blade (e.g., Accu-Edge, Sakura Finetek, Cat# 4689, Sakura Finetek, Torrance, CA, USA) to the apex of the tissue using a paraffin microtome (e.g., Leica 2155 rotary paraffin microtome, Leica microbiosystems, Wetzlar, Germany).

Note: Samples should be kept on ice between sectioning. Colder blocks will provide cleaner sections. Similarly, a 40–42 °C water bath should be used to collect sections. When the dehydrated tissue comes into contact with water the tissue will begin to expand. Embedded adipocytes are fragile. Therefore, if the water is too warm the tissue will expand quickly tearing the delicate adipocyte membrane and leading to difficulties with downstream analysis.

Starting at the tissue apex $5 \times 5 \mu\text{m}$ sections are made at a minimum of 100 μm intervals across the sample tissue. Using a minimum interval distance of 100 μm will ensure that each section will contain a unique sampling of adipocytes. Serial sections are recommended as this will provide matching tissue samples for additional independent analyses (e.g., immunohistochemistry). Sections are placed onto labeled electrostatically charged precleaned microscope slides (e.g., Cat# 12-550-15, Thermo Fisher Scientific, Waltham, MA, USA) and are left to dry for a minimum of 24 h.

1. Embedded tissues are faced off to tissue apex
2. $5 \times 5 \mu\text{m}$ sections are taken at 100 μm intervals and placed on slides
3. Slides are allowed to dry for 24 h

Note: Cutting tissue seems like an inconsequential process, however paraffin-embedded adipose tissue is very delicate and membranes are prone to tearing. Extreme caution should be taken with sectioning. It is wise to perfect sectioning on nonessential adipose tissue samples before moving onto important samples.

Once dry, slides are placed into a 60 °C dry oven for 1.5 h to begin removal of paraffin, and any residual water. Tissues are then stained with H&E using the following method.

1. Slides are baked at 60 °C for 1.5 h
2. 5 min in xylene ($\times 2$)
3. 2 min in 100% ethanol ($\times 2$)
4. 2 min in 95% ethanol ($\times 2$)
5. 2 min in 70% ethanol
6. 2 min in deionized H_2O
7. Stain for 2 min in Harris' hematoxylin (Cat# HHS16, Sigma-Aldrich, St. Louis, MO, USA)

8. Rinse under running H₂O until clear
9. Three dips in a bluing solution
10. Rinse for 2 min under gently running H₂O
11. 2 min in 80% ethanol
12. 2 min in eosin (Cat# 318906, Sigma-Aldrich, St. Louis, MO, USA)
13. Four dips in 80% ethanol to rinse out excess eosin
14. 2 min in 95% ethanol
15. 2 min in 95% ethanol
16. 2 min in 100% ethanol (×2)
17. 3 min in xylene (×3)

Slides are then treated with a xylene-based permount and coverslips mounted (e.g., Cat# 2955-245, Corning Incorporated, Corning, NY, USA). Slides should be left horizontal to set for at least 24 h before putting them into a storage box.

2.2. Image collection and analysis

2.2.1 Using MetaMorph microscopy automation and image analysis software

—Using a Zeiss inverted fluorescent microscope (or similar) five representative photos of each section are taken with a 40 × objective using a monochrome AxioCAM MrC (or similar) camera. A semiautomated custom image analysis protocol was developed using MetaMorph image analysis software to quantify the area of the individual adipocytes. A brief description of the MetaMorph program is as follows. Based on the magnification of the microscope used, each image is first calibrated to a known distance. For a Zeiss microscope at 40 × objective this equates to 0.6812 μm per pixel. Adipocyte cell membranes are enhanced by 3 pixels through erosion filtration to improve the cell boundaries. Histological images are segmented by an inclusive threshold filtration (200 low and 255 high) creating a binary mask and converting the image to a 1-bit configuration. Any adipocytes with visible lacerations to the membranes are “closed” digitally prior to continuing with the automated area quantification. To quantify the adipocyte areas, images are analyzed and adipocytes highlighted if they meet the following four criteria: (1) the adipocyte contains an area between 500 and 15,000 μm²; (2) the adipocyte has a shape factor of 0.35–1 (a shape factor of 0 indicating a straight line and 1 a perfect circle); (3) the adipocyte has an equivalent sphere surface area between 5,000 and 1,000,000 μm²; and (4) the adipocyte does not border the image frame. In general, 70% of total tissue adipocytes meet these criteria and are picked up automatically. An additional 10% of adipocytes meet the aforementioned criteria, but are not picked up by the automated program. Thus for our studies, size of these adipocytes was included after manually. To visualize the adipocytes more clearly and provide traceability for their respective quantified areas, each adipocyte is subsequently labeled with a unique number. The unique adipocyte number and respective area is then exported for statistical analysis.

The following is a detailed description of setting up and running the MetaMorph program (Molecular Devices, Sunnyvale, CA, USA) with the adipocyte analysis tool. To run this analysis both MetaMorph and Microsoft Excel (Microsoft, Albuquerque, NM, USA) software programs will be needed.

1. Open base image

In the tool bar located at the top of the screen File→Open are selected. An image of interest is then chosen.

2. Calibrate image(s).

The first step in analyzing the adipocytes is to calibrate the image. The process will allow the program to recognize the size of the sample based on the number of pixels and the magnification of the microscope. The following steps are applied for each image.

Located in the tool bar at the top of the screen Measure→Calibrate Distances are chosen. This opens a “Calibration Distances” window. In the Calibration box the correct calibration name is selected. To create a new calibration, see the instructions below. “Apply to All Open Images” button is subsequently chosen.

How to add a new calibration

An image with scale bar of a known length is opened. This image should be taken at the same magnification as the adipocyte of interest. The “+” button is selected. A new entry line item called “Calibration #x” appears in the Calibration box. The line item is selected and the “Calibrate by Region” button clicked. A new window appears called “Calibrate by Region.” The steps in *BLUE* are followed. The image containing a stage micrometer or other calibration standard is selected. It should be listed in the “Image” box. If not, the “Image” box is clicked and the correct image is chosen. The “Type of Region Tool” is chosen. This will allow the calibration standard on the image to be measured. The calibration is defined by choosing “Line Rgn” (not “Rectangle Rgn”). The region position and size is adjusted to determine the calibration ratio of selected units to pixels. In the calibration window a box labeled with “Name of Calibration” (e.g., Calibration #x) will be present. A descriptive name (e.g., Zeiss 40 × AxioCam MrC, etc.) can be provided in the “Name of Calibration” box. Because each of the future images is taken at the same magnification and on the same microscope, this calibration can be used for future analyses. If any of the imaging variables change then a new calibration must be made. A value will need to be entered into the X box (Y: if rectangle) of the “Calibrated Length” window. This value is determined by the standard scale bar in the image. Typically, this will be in the micron range. The “Region Length X” (Size if rectangle, Y: if rectangle) is set by the length (in pixels) of the standard scale bar. The region is measured by clicking and holding the middle of the calibration line. The calibration line is moved so that one edge is at the start of the scale bar. The start or end of the line is measured by clicking/holding on the ends (little squares) and moving them to the desired location. The line is positioned to travel straight across the scale bar. The “Units/Pixel X” (Y: if rectangle) box will

display a value for the units per pixel based on the calibrated length that was entered and the actual length of the standard scale (in pixels). For the “Units” box, the correct units are chosen by clicking on the down arrowhead. When finished, these calibrations are ready to be used by pressing “OK.” These calibrations are applied to all current and future images.

3. Enhance border

To help define the membranes of the individual adipocytes each membrane was enhanced by 3 pixels. The benefit of this enhancement materializes in steps 6 and 7 when the computer algorithm identifies the individual adipocytes. This process is accomplished via the following steps.

Located within the tool bar Process→Morphological Filters→Erode→Parameters menu are selected. The following parameters must be specified.

- Filter shape: click on circle
- Diameter: 3 pixels

Once these are adjusted the APPLY button is pressed. A new Image appears named “Erode.” If the “Erode” button is clicked a preferred name can be entered (Specified(Erode)) before pressing APPLY.

4. Threshold image

In this step, the images are transformed into a binary image. The purpose is to provide defined areas consisting of membrane material and empty space identified by black and white respectively. This process is accomplished via the following steps.

Located within the tool bar, Measure→Threshold Image menu is selected. The eroded image must be listed in the “Source Image” box. If it is not it can be changed by clicking on the “Source Image” and changing it to “Erode.” The following parameters must also be changed. In the threshold tab the “Threshold State” should be set to inclusive. The low level should be set from 0 to high (255) so that orange color fills the white spaces. Depending on the clarity of the image these numbers may change. Keep in mind that the orange will fill the areas that are empty spaces where the lipid would be found in the adipocytes. Adjustments should be made with the low/high level to best fit the threshold to the empty space.

Next in the clip tab, the “Create Binary Mask” found in the mode section is selected. The bit depth of binary mask should then be set to 1-bit. The resulting image will be called “1-Bit Binary.” If the name “1-Bit Binary” is clicked the name can be adjusted to whatever is preferred (Specified(1-Bit Binary)) before pressing APPLY. A new image appears named “1-Bit Binary” (or the selected name).

5. Separate unique adipocytes

This is a good place for the “Cut Drawing” tool to be used to separate adipocytes that have a perforated membrane and are joined to a second adipocyte. The “Cut Drawing Tool” is used (the icon looks like a pencil with red tip down) to separate

two or more joined adipocytes with a black line. The “Cut Drawing Tool” is selected and then in the “1-Bit Binary” image, the left mouse button is clicked and held while a small line is used to “cut” the object into separate parts. This process is repeated as necessary.

6. Define initial areas of interest

Now that the adipocytes are defined, a computer algorithm can be used to identify the unique areas of the individual adipocytes. To accomplish this step the following steps are followed:

Located in the tool bar at the top of the screen Measure→Integrated Morphometry Analysis is opened and the Source: “1-Bit Binary” is selected.

The following characteristics should be marked off in the “Preferences” tab:

- Measure methods: Check “Measure All Regions,” “Fill Holes in Objects” and “Exclude Objects Touching Edge.”
- Object Drawing: Check “Draw Object Borders” and “Draw Centroid Mark.”
- Miscellaneous: NONE of the options should be checked.
- Mouse click interaction: Check “Highlight Objects on Left Click” and “Add/Remove Object from Measurement Set on Double Click.”
- Object standards: “Standard area: 1,” “Optical Density Low Boundary: 1,” “Optical Density High Boundary: 255.”
- Object mask: Check “Binary-Intensity of 1 for each Object.”

The following should be marked off in the “Measurement Parameters and Filters” tab.

- Area: “Display and Filter” are checked. “Comparison” is between Limit 1:500 and Limit 2:15000.
- Shape (Shape Factor): “Display and Filter” are checked. “Comparison” is between Limit 1:0.35 and Limit 2:1.000.
- Equiv (Equiv. sphere surface area): “Display and Filter” are checked. “Comparison”: between Limit 1:5000, Limit 2:1,000,000.

The “Reset Current” button is subsequently pressed prior to clicking on the “Measure” button. The “1-Bit Binary” image will have an object overlay appear that colors objects green. When an object is clicked it will be highlighted in yellow. In the “Object Tab,” you can see the area, shape and equivalence values for the selected object.

7. Visualize objects more clearly by creating numbered regions over the object

To provide traceability each adipocyte is labeled with a unique number. To accomplish this, the following process is followed:

The “Region Tool Properties” is selected. The icon looks like an overlapping combination of a red circle, red square, notepad and pencil located in tool bar. For the “Region color” NOTHING SHOULD BE CHECKED especially “Use Same Region for New Regions.”

On the tool bar Edit→Preferences is opened.

- In the “Region Labels” tab “Draw Labels Next to Regions” should be checked.
- For the label location “Center” should be checked.
- “Draw Label Inside of Region Boundary” as well as “Draw Labels With Respect to the Image Zoom” are also checked.
- For the label color “Same as Region” is checked prior to pressing OK.

Now located in the tool bar Regions→Create Regions Around Objects is chosen.

This will put numbered regions around the objects to help identify which objects need editing.

A moment should be taken to look at the image. If it appears that there are adipocytes that are joined together that should in fact be two or more unique cells then proceed through step 8 or 9 by first removing regions (for now) by clicking Regions→Clear Regions→Yes.

If on the other hand adipocytes identified appear accurate continue to step 10.

8. Modify selected adipocytes by splitting a continuous adipocyte into smaller parts

If an identified adipocyte is labeled as one continuous adipocyte but should in fact be 2 or more, the adipocytes can be separated using the following steps. The object that is not restricted to a defined adipocyte boundary is selected. If the yellow highlight covers two or more adipocytes then it will need to be split using the “Cut Drawing Tool” (Icon looks like a pencil with red tip down). The “Cut Drawing Tool” is chosen and then in the “1-Bit Binary” image, the left mouse button clicked and held while a small black line is drawn to “cut” the object into separate parts.

The “Reset Current” button is pressed followed by the “Measure button.” The “1-Bit Binary” image will have an object overlay appear that colors objects in green. The object can be selected to see if the object was successfully clipped in two. If not, the procedure is repeated again.

9. Modify selected objects by joining a split object into a larger object

If a single adipocyte is being registered as two separate adipocytes they can be joined together manually. Click on an object/adipocyte that you feel is broken into smaller parts. If the yellow highlight covers a fragment of the adipocyte, then the fragments are joined by using the “Join Drawing Tool” (icon looks like a pencil with red tip up). The “Join Drawing Tool” is clicked and then in the “1-Bit Binary” image, the left mouse button is pressed and held while drawing a small red line between the fragments to “join” them into one larger object. The “Reset Current”

button is then selected followed by the “Measure” button. The “1-Bit Binary” image will have an object overlay appear that colors objects in green. The object can be chosen to confirm the adipocytes have been combined successfully. If not, the procedure is repeated again.

10. Adding/removing objects that are not highlighted in green

If the automatic computer algorithm does not register a specific adipocyte, it can be added manually by double left-clicking rapidly on the object that is not included and highlighted in green. If, on the other hand, the program identifies an object that is not an adipocyte it can be removed by similarly double left-clicking rapidly on the object.

Note: Be patient between double clicks, it may take a moment for the new object to become highlighted/unhighlighted.

11. Logging data

Once the image has been analyzed, the associated data need to be recorded. Recording the data is done by logging the data from objects.

Data can be logged as objects in the Integrated Morphometry Analysis Window. The “Object Data” Tab is selected followed by the “Configure Log” button. A new window will open called “Configure Log.” The “Parameter Configurations” is opened to select the parameters that will appear in the Excel spreadsheet. Examples include image name, object number, area, shape factor, equiv. sphere surface area, etc. In the “Logging Options” tab, “Log Column Titles” is checked followed by OK. Next the “Open Log” button is chosen. A new window will open called “Open Object Log.” In the “Log Measurements” section “Dynamic Data Exchange (DDE)” is pressed followed by OK. A new window will open called “Export Log Data.” For the application, “Microsoft Excel” is chosen. The sheet name (typically Sheet1), starting row (typically 1) and starting column (typically 1) are selected, followed by OK. Excel will open and you will be taken to the Excel program. Click on “MetaMorph Offline Button” in the Task bar to return to MetaMorph. The Open Log button is now named “F9: Log Data.” Press the “F9: Log Data Button” and the object data will be pasted into Excel. The Excel sheet is SAVED at this point to prevent data loss.

Once the data have been logged, Excel can be disconnected from MetaMorph by closing the Excel Sheet and/or Excel program. By doing so a new window appears in MetaMorph called “Link Disconnected.” By pressing on “OK” Excel becomes disconnected from MetaMorph. To log any further data via this method, a connection between Excel and MetaMorph will need to be re-established.

12. Saving regions

Regions can be saved for later viewing by selecting Select Regions→Save Regions from the task bar. A new window appears to save regions to a folder. These regions can be opened at a later date by selecting Regions→Load Regions. The correct image should be opened before loading regions.

13. Adding regions manually

A region can be added manually by clicking on the “Trace Region Icon.” This icon looks like an irregular shaped object in the tool bar on top panel. In the image, the first point of the adipocyte parameter is anchored by left-clicking once. The outer parameter of the adipocyte is then traced by moving along the outer edge and anchoring points by left-clicking. When the entire parameter is traced double left-clicking the mouse completes the identification of the region.

Note: The tool will stay on the “Trace Region Icon” unless an option is selected under the “Region Tool Properties.” This can be removed by clicking on the Icon that looks like a combination of a “red circle, red square, notepad and pencil” in the tool bar and unchecking the box in the right-hand side that says “Revert to Locator Tool” after creating a region.

14. Logging data for one active region at a time

Individual active regions are logged by clicking Measure→Region Measurements in the task bar. A new window called “Region Measurements” will open. The correct file should be confirmed as listed in the box on the top of the region measurement window. In the “Include” tab “Select Active Region” is chosen from the pull down menu. The parameters of interest, for example, Image name, Object Number, Area, Shape factor, Equiv. sphere surface area are chosen from the “Configure Tab.” The following parameters are also selected.

- In the “Color Channel,” “Intensity” is selected.
- In the “Display and Log” section, “Region Measurements and Summary” is checked.
- “Log Image Calibration” and “Log Column Titles” are checked.
- “Use Threshold for Intensity Measurements” is NOT checked.

Once these parameters are set, the “Measurement” tab is selected followed by the “Open Log” button. A new window opens called “Open Object Log.” In the “Log Measurements to Section,” “DDE” is chosen followed by “OK.” A new window opens called “Export Log Data.” Microsoft Excel is chosen for the program. The Sheet (typically Sheet1), Starting Row (typically 1), and Starting Column (typically 1) are all named followed by selecting “OK.” Excel will open. MetaMorph Offline Button is chosen in the Task bar to return to MetaMorph. The “Open Log” button is now named “F9: Log Data.” The “F9: Log Data Button” is selected and the object data are subsequently pasted into Excel.

Traced regions can be saved by selecting Select Regions→Save Regions from the tool bar. A new window appears to save regions to a folder. These regions can be opened at a later date by selecting Regions→Load Regions. The correct image must be opened prior loading the regions.

15. Removing unwanted regions.

Unwanted regions can be removed by clicking on the regions edge. A flickering animated outline of the adipocyte will indicate it has been selected. Delete button is then pressed on the keyboard to remove the region.

16. Making a screen shot of the image with numbered regions

Once the image is displaying all of the desired information, such as labeled regions, etc. (see steps 12–14 above to make regions or load regions into image) Edit→Duplicate→As Displayed is selected. A new window appears called “Duplicate As Displayed”. Making sure the correct image is in the box under “Source Image: Destination Image,” “Duplicate: Select Entire Image” is selected followed by “OK.” The image is then saved by clicking on File→Save As. A new window appears “Save File Name As.” A folder and name are chosen and saved as a MetaSeries Single/Multi-plane TIFF (*.tif). The Default name for all images is “Untitled.” The name can be changed by clicking on the “Untitled” Button. Alternatively the image can be saved, by right-clicking in the “Duplicate as Displayed Image” and copied by selecting “Copy Image to Clipboard.” This can be pasted into an Excel spreadsheet.

Steps 1–16 are repeated for all subsequent images of interest.

2.2.2 Using ImageJ image analysis software—Although using the MetaMorph semiautomated software is the most efficient way of analyzing the adipocyte histology, the price of the software is limiting for some laboratories. Accordingly, a similar manual procedure has been developed using the free open access software ImageJ (<http://rsbweb.nih.gov/ij/>). It should be noted that this method will be slightly slower as it requires manual identification of the adipocytes.

1. Open base image

The image of interest is opened by clicking on File→Open in the task bar.

2. Duplicate image

The image is duplicated by clicking the right button of the mouse on the image of interest and selecting “Duplicate Image.” The name of the image is chosen.

3. Calibrate image(s)

The first step in analyzing the adipocytes is to calibrate the image. The process allows the program to recognize the size of the sample based on the number of pixels and the magnification of the microscope. For each image the following steps are applied.

On the tool bar Analyze→Set scale is selected. The following parameters are set and are dependent on the magnification of the image and the camera settings.

- Distance in pixels
- Known distance
- Pixel aspect ratio = 1 (for most digital cameras)

- Unit of length = μm

To determine these parameters an image with a known linear scale bar is opened. It is important that this image is taken at the same magnification as your images of interest. The “straight line” tool is selected on the tool bar. A line is drawn over the scale bar on the image, keeping the line as straight as possible. Analyze→Set Scale is selected from the task bar, opening the set scale window. The distance that was measured in pixels is displayed in the “Distance in Pixels Box.” The known distance of the linear scale bar is entered into the “Known Distance Box.” The pixel aspect ratio for most cameras is set to 1. The unit of length is dependent on the scale bar and is likely in microns. The “Global” box is selected to maintain the settings for all subsequent image analysis. Once the calibration settings are known they can be used for all subsequent images taken at the same magnification using the same camera.

4. Subtract background

To help clarify the image for subsequent analysis, the backgrounds of images are removed based on a “rolling ball” algorithm (Sternberg, 1983). To process the image Process→Subtract Background is selected from the task bar. The following parameters should be set.

- “Rolling Ball Radius” should be set to 50 pixels
- “Light Background” should be checked
- “Create Background (do not subtract)” should NOT be checked
- “Sliding Paraboloid” should be checked
- “Disable Smooth” should NOT be checked
- “Preview” should be checked

When all of the parameters are set “OK” is selected.

5. Remove excess noise

Often times when photos of tissues are taken random variations in the brightness or color information occur. This “noise” is removed by selecting Process→Noise→Despeckle in the tool bar.

6. Threshold images

The purpose of this step is to provide defined areas consisting of membrane material and empty space identified by black and white respectively. This process is accomplished via the following steps: Image→Adjust→Threshold are selected from the tool bar. The “Threshold Type” should be set to “Default” as well as “Over/Under.” The box labeled “Dark Background” is selected. The first sliding bar should be set to 200 (this may vary depending on the clarity of the image) while the second sliding bar will be set to 255. Once these parameters are set “Apply” is chosen.

Note: The blue highlight should maximally cover the areas defined as the adipocyte membranes without covering the empty space that would normally contain lipid.

7. Convert image to a binary format

Now that the areas of interest are defined, the image needs to be converted to a binary format to allow for future analysis. To convert the image Process→Binary→Make Binary are selected from the tool bar.

Note: This process will convert the dominantly black image to a dominantly white image.

8. Enhance border

To help define the membranes of the individual adipocytes each membrane is uniformly enhanced. The benefit of this enhancement materializes in step 8 when identifying the areas of the individual adipocytes. To enhance the border: Process→Binary→Dilate are chosen.

Note: The processing of the images (Steps 4–8) can be automated by creating a macro. Plugins→New→Macro is selected and a new screen will appear. The following computer script is subsequently copied and pasted into this window.

```
run("Subtract Background...", "rolling=50 light sliding");
run("Despeckle");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(200, 255);
run("Convert to Mask");
run("Make Binary");
run("Dilate");
```

This macro should be subsequently named and saved (File→Save). Once this macro is created, images can be easily analyzed by simply opening and calibrating the image, then by running the macro (click on Plugins→Macros→Run, opening the saved macro).

9. Measure and label adipocytes

To measure the area of the adipocytes you will need to download the free “Measure and Label Macro” for imageJ (<http://rsbweb.nih.gov/ij/plugins/measure-label.html>). This macro will provide a read out of the adipocyte area and place a corresponding unique number within the center of the adipocyte. This method of measuring the area has the benefit of providing traceability for each adipocyte. Once the macro has been downloaded and installed, create a short cut key to activate the macro program. To initiate on the macro Plugin→Shortcuts→Create Shortcuts is selected in the tool bar. The “Measure and Label Macro” is chosen in the command pull down menu. A key (that is not already set, e.g., q) needs to be selected to be set as the shortcut. The “Wand” (tracing tool) located in the tool bar is subsequently chosen. Using this tool, the individual adipocytes of interest are clicked on. A yellow tracing of the inside of the adipocyte will appear. The shortcut key (e.g., q) that you assigned to the “Measure and Label Macro” is then clicked. A unique

number will be placed in the center of the adipocyte, and in a pop-up results screen the corresponding area will be appear. The “Wand” tool and macro are then iteratively used until all remaining adipocytes are counted.

Note: Do not count adipocytes that are touching the border of the image.

Once all of the adipocytes have been counted, the recorded data are copied by clicking on the right button of the mouse, select all, then on copy, then pasting it into an excel spreadsheet. By clicking on File→Save as→Tiff this image can be saved for future reference.

Repeat steps 1 through 8 for all of the individual images of interest.

2.3. Data analysis

Once the areas of the adipocytes of interest are determined, a frequency distribution is calculated for a given tissue, being sure to first remove any objects that fall below an area of $350 \mu\text{m}^2$, as these cells may be a mixture of adipocytes and stromal vascular cells. The frequency can be accomplished easily using the frequency function in Excel (`=frequency(data_array, bins_array)`). The size of the array bins may vary depending on the animal model; however, a distribution from 0 to $15,000 \mu\text{m}^2$ in 500 increments is appropriate for most WAT depots. The number of total adipocytes within the distribution is subsequently calculated and used to convert the frequency to a percentage of total adipocytes counted (Fig. 6.2A). The benefit of using a frequency distribution rather than the average adipocyte area alone emerges when there is a change in adipocyte number in addition to size. In this case, while the overall average area of the adipocytes may not change, the number of adipocytes of a given size may be altered. By providing a frequency distribution these types of changes are accounted for. A comparison between two frequencies is made using a two-way ANOVA followed by a Bonferroni post hoc analysis. To ascertain whether there is a change in adipocyte number and size between animal models, a correlation between the average adipocyte volume and the weight of the adipose depot is determined. To calculate the average adipocyte volume, the radius of each individual adipocyte area is determined, assuming a circular model. This radius is used to calculate the volume (assuming a spherical model) of the given adipocyte. The average volume of a given tissue is then calculated from these volumes. A linear correlation with a two-tailed comparison of slope and intercept is calculated and compared between different treatment groups (Fig. 6.2B). Analysis of adipocyte cellularity can then be made using either MetaMorph or ImageJ as both provide equivalent distributions (Fig. 6.3A) and average adipocyte size (Fig. 6.3B).

2.4. Supporting data, results, and experimental guidance

A number of factors need to be taken into consideration when performing quantitative histomorphometry on adipose tissue. As was mentioned above, significant differences exist between the expression profile of adipocyte genes between tissues and within tissues (Brochu et al., 2001; Fujimoto et al., 1999; Klöting et al., 2010; Lau et al., 2011; Lefebvre et al., 1998; Nicklas et al., 2004). Analysis of adipocyte size using our described method has indicated that there are differences in the distribution of adipocyte sizes and average area

between inguinal and epididymal WAT (Fig. 6.4). In contrast, with the exception of adipose tissue located directly on the testis, there were no differences in the distribution of adipocyte size or average area across the epididymal tissue depot (Fig. 6.5). As a consequence, when carrying out a comparison between treatments or genotypes in murine animal models, it is important that the subcutaneous and visceral adipose depots are considered separately and specimens taken from epididymal adipose depots should not be sampled directly from the testes.

In addition to the specific depot and location, the number of adipocytes counted and the number of animals used in the experiment are important considerations. While counting 5000 adipocytes per animal (Fig. 6.2) is possible and provides an extremely accurate distribution of the adipocyte areas across the tissue bed, it is a product of diminishing returns. By comparing the average area calculated from 3, 10, 100, 300, 500, 1000 randomly selected adipocytes repeated 10 times from an individual animal, we identified three adipocytes as the lowest evaluated number required to accurately predict average adipocyte area (Fig. 6.6A); however, counting 100 adipocytes is required to achieve a reasonably small variance around that mean (Fig. 6.6B). Counting 100 adipocytes also results in a similar distribution of adipocyte sizes compared to 300, 500, and 1000 adipocytes (Fig. 6.6C). Accordingly, our data suggest that as few as 100 adipocytes need to be counted to obtain an accurate estimation of adipocyte sizes within an adipose tissue depot. Finally, by using the frequency distribution (Fig. 6.7A) or volume of adipocytes (Fig. 6.7B) in the epididymal adipose depot, the mean adipocyte radius was calculated for mice fed a standard chow ($26.7 \pm 3.1 \mu\text{m}$) or high-fat ($33.8 \pm 3.9 \mu\text{m}$) diet. Based on these variances we estimated using the power equation ($1 - \beta = 0.8$, $\alpha = 0.05$) (National Research Council, 2003) that a minimum of five animals per treatment group would be required to see a $\sim 7 \mu\text{m}$ difference from the observed mean radius (Fig. 6.7C and D), which would be comparable to the difference in average radius between the chow and high-fat diet groups.

3. FUTURE CHALLENGES AND CONCLUSIONS

While our method provides an easy and semiautomated protocol for quantitatively analyzing size and number of adipocytes within adipose tissue at a given time, this approach does not estimate rate of adipocyte differentiation or death within the tissue. Adipose tissue is in a constant state of flux with 1–5% of mouse adipocytes turning over daily (Rigamonti, Brennand, Lau, & Cowan, 2011) and $\sim 8\%$ of human adipocytes turning over every year (Spalding et al., 2008). Consequently, in addition to changes in adipocyte size and number, a future challenge will be to incorporate an easy method to evaluate rate of formation and turnover of adipocytes. However, the method described herein should have utility for both basic and clinical research, given the tremendous interest in the development, metabolism, and endocrine functions of adipose tissue development under physiological and pathophysiological conditions.

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REFERENCES

- Ailhaud G, Grimaldi P, Negrel R. Cellular and molecular aspects of adipose tissue development. *Annual Review of Nutrition*. 1992; 12:207–233. <http://dx.doi.org/10.1146/annurev.nu.12.070192.001231>.
- Bjorndal B, Burri L, Staalesen V, Skorve J, Berge RK. Different adipose depots: Their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. *Journal of Obesity*. 2011; 2011:490650. <http://dx.doi.org/10.1155/2011/490650>. [PubMed: 21403826]
- Bjornheden T, Jakubowicz B, Levin M, Oden B, Eden S, Sjoström L, et al. Computerized determination of adipocyte size. *Obesity Research*. 2004; 12(1):95–105. <http://dx.doi.org/10.1038/oby.2004.13>. [PubMed: 14742847]
- Bradshaw AD, Graves DC, Motamed K, Sage EH. SPARC-null mice exhibit increased adiposity without significant differences in overall body weight. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(10):6045–6050. <http://dx.doi.org/10.1073/pnas.1030790100>, PII: 1030790100. [PubMed: 12721366]
- Brochu M, Tchernof A, Dionne IJ, Sites CK, Eltabbakh GH, Sims EA, et al. What are the physical characteristics associated with a normal metabolic profile despite a high level of obesity in postmenopausal women? *The Journal of Clinical Endocrinology and Metabolism*. 2001; 86(3): 1020–1025. [PubMed: 11238480]
- Casteilla L, Penicaud L, Cousin B, Calise D. Choosing an adipose tissue depot for sampling: Factors in selection and depot specificity. *Methods in Molecular Biology*. 2008; 456:23–38. http://dx.doi.org/10.1007/978-1-59745-245-8_2. [PubMed: 18516550]
- Cawthorn WP, Scheller EL, MacDougald OA. Adipose tissue stem cells meet preadipocyte commitment: Going back to the future. *Journal of Lipid Research*. 2012a; 53(2):227–246. <http://dx.doi.org/10.1194/jlr.R021089>. [PubMed: 22140268]
- Cawthorn WP, Scheller EL, MacDougald OA. Adipose tissue stem cells: The great WAT hope. *Trends in Endocrinology and Metabolism*. 2012b; 23(6):270–277. <http://dx.doi.org/10.1016/j.tem.2012.01.003>. [PubMed: 22417866]
- Chen HC, Farese RV Jr. Determination of adipocyte size by computer image analysis. *Journal of Lipid Research*. 2002; 43(6):986–989. [PubMed: 12032175]
- Cinti S. The adipose organ. Prostaglandins, Leukotrienes, and Essential Fatty Acids. 2005; 73(1):9–15. <http://dx.doi.org/10.1016/j.plefa.2005.04.010>, PII: S0952-3278(05)00054-2.
- de Ferranti S, Mozaffarian D. The perfect storm: Obesity, adipocyte dysfunction, and metabolic consequences. *Clinical Chemistry*. 2008; 54(6):945–955. <http://dx.doi.org/10.1373/clinchem.2007.100156>, PII: clinchem.2007.100156. [PubMed: 18436717]
- Desruisseaux MS, Nagajyothi, Trujillo ME, Tanowitz HB, Scherer PE. Adipocyte, adipose tissue, and infectious disease. *Infection and Immunity*. 2007; 75(3):1066–1078. <http://dx.doi.org/10.1128/IAI.01455-06>. [PubMed: 17118983]
- Faust IM, Johnson PR, Stern JS, Hirsch J. Diet-induced adipocyte number increase in adult rats: A new model of obesity. *The American Journal of Physiology*. 1978; 235(3):E279–286. [PubMed: 696822]
- Fox CH, Johnson FB, Whiting J, Roller PP. Formaldehyde fixation. *The Journal of Histochemistry and Cytochemistry*. 1985; 33(8):845–853. [PubMed: 3894502]
- Fujimoto WY, Bergstrom RW, Boyko EJ, Chen KW, Leonetti DL, Newell-Morris L, et al. Visceral adiposity and incident coronary heart disease in Japanese-American men. The 10-year follow-up results of the Seattle Japanese-American Community Diabetes Study. *Diabetes Care*. 1999; 22(11):1808–1812. [PubMed: 10546012]
- Gerin I, Dolinsky VW, Shackman JG, Kennedy RT, Chiang SH, Burant CF, et al. LXRbeta is required for adipocyte growth, glucose homeostasis, and beta cell function. *The Journal of Biological Chemistry*. 2005; 280(24):23024–23031. <http://dx.doi.org/10.1074/jbc.M412564200>. [PubMed: 15831500]

- Gerin I, Louis GW, Zhang X, Prestwich TC, Kumar TR, Myers MG Jr. et al. Hyperphagia and obesity in female mice lacking tissue inhibitor of metalloproteinase-1. *Endocrinology*. 2009; 150(4):1697–1704. <http://dx.doi.org/10.1210/en.2008-1409>. [PubMed: 19036876]
- Goralski KB, Sinal CJ. Type 2 diabetes and cardiovascular disease: Getting to the fat of the matter. *Canadian Journal of Physiology and Pharmacology*. 2007; 85(1):113–132. <http://dx.doi.org/10.1139/y06-092>, PII: y06-092. [PubMed: 17487251]
- Han J, Lee JE, Jin J, Lim JS, Oh N, Kim K, et al. The spatiotemporal development of adipose tissue. *Development*. 2011; 138(22):5027–5037. <http://dx.doi.org/10.1242/dev.067686>. [PubMed: 22028034]
- Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ, Martin RJ. The biology of white adipocyte proliferation. *Obesity Reviews*. 2001; 2(4):239–254. [PubMed: 12119995]
- Hirsch J, Gallian E. Methods for the determination of adipose cell size in man and animals. *Journal of Lipid Research*. 1968; 9(1):110–119. [PubMed: 4295346]
- Kaartinen JM, LaNoue KF, Martin LF, Vikman HL, Ohisalo JJ. Beta-adrenergic responsiveness of adenylate cyclase in human adipocyte plasma membranes in obesity and after massive weight reduction. *Metabolism*. 1995; 44(10):1288–1292. PII: 0026-0495(95)90031-4. [PubMed: 7476286]
- Kloting N, Fasshauer M, Dietrich A, Kovacs P, Schon MR, Kern M, et al. Insulin-sensitive obesity. *American Journal of Physiology Endocrinology and Metabolism*. 2010; 299(3):E506–515. <http://dx.doi.org/10.1152/ajpendo.00586.2009>, PII: ajpendo.00586.2009. [PubMed: 20570822]
- Lau FH, Deo RC, Mowrer G, Caplin J, Ahfeldt T, Kaplan A, et al. Pattern specification and immune response transcriptional signatures of pericardial and subcutaneous adipose tissue. *PLoS One*. 2011; 6(10):e26092. <http://dx.doi.org/10.1371/journal.pone.0026092>, PII: PONE-D-11-00779. [PubMed: 22022522]
- Lee YH, Chen SY, Wiesner RJ, Huang YF. Simple flow cytometric method used to assess lipid accumulation in fat cells. *Journal of Lipid Research*. 2004; 45(6):1162–1167. <http://dx.doi.org/10.1194/jlr.D300028-JLR200>. [PubMed: 14993237]
- Lee MJ, Pramyothin P, Karastergiou K, Fried SK. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. *Biochimica et Biophysica Acta*. 2013 pii: S0925–4439(13)00191–9. <http://dx.doi.org/10.1016/j.bbadis>.
- Lefebvre AM, Laville M, Vega N, Riou JP, van Gaal L, Auwerx J, et al. Depot-specific differences in adipose tissue gene expression in lean and obese subjects. *Diabetes*. 1998; 47(1):98–103. [PubMed: 9421381]
- Le Lay S, Krief S, Farnier C, Lefrere I, Le Liepvre X, Bazin R, et al. Cholesterol, a cell size-dependent signal that regulates glucose metabolism and gene expression in adipocytes. *The Journal of Biological Chemistry*. 2001; 276(20):16904–16910. <http://dx.doi.org/10.1074/jbc.M010955200>, PII: M010955200. [PubMed: 11278795]
- Longo KA, Wright WS, Kang S, Gerin I, Chiang SH, Lucas PC, et al. Wnt10b inhibits development of white and brown adipose tissues. *The Journal of Biological Chemistry*. 2004; 279(34):35503–35509. <http://dx.doi.org/10.1074/jbc.M402937200>. [PubMed: 15190075]
- Maroni BJ, Haesemeyer R, Wilson LK, DiGirolamo M. Electronic determination of size and number in isolated unfixed adipocyte populations. *Journal of Lipid Research*. 1990; 31(9):1703–1709. [PubMed: 2246619]
- Mori H, Prestwich TC, Reid MA, Longo KA, Gerin I, Cawthorn WP, et al. Secreted frizzled-related protein 5 suppresses adipocyte mitochondrial metabolism through WNT inhibition. *The Journal of Clinical Investigation*. 2012; 122(7):2405–2416. <http://dx.doi.org/10.1172/JCI63604>. [PubMed: 22728933]
- National Research Council. Guidelines for the care and use of mammals in neuroscience and behavioral research. The National Academies Press.; Washington: 2003.
- Nicklas BJ, Penninx BW, Cesari M, Kritchevsky SB, Newman AB, Kanaya AM, et al. Association of visceral adipose tissue with incident myocardial infarction in older men and women: The Health, Aging and Body Composition Study. *American Journal of Epidemiology*. 2004; 160(8):741–749. <http://dx.doi.org/10.1093/aje/kwh281>, PII: 160/8/741. [PubMed: 15466496]

- Nishimura S, Manabe I, Nagasaki M, Hosoya Y, Yamashita H, Fujita H, et al. Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. *Diabetes*. 2007; 56(6):1517–1526. <http://dx.doi.org/10.2337/db06-1749>. [PubMed: 17389330]
- Okamoto Y, Higashiyama H, Inoue H, Kanematsu M, Kinoshita M, Asano S. Quantitative image analysis in adipose tissue using an automated image analysis system: Differential effects of peroxisome proliferator-activated receptor- α and - γ agonist on white and brown adipose tissue morphology in AKR obese and db/db diabetic mice. *Pathology International*. 2007; 57(6): 369–377. <http://dx.doi.org/10.1111/j.1440-1827.2007.02109.x>, PII: PIN2109. [PubMed: 17539968]
- Osman OS, Selway JL, Kepczynska MA, Stocker CJ, O'Dowd JF, Cawthorne MA, et al. A novel automated image analysis method for accurate adipocyte quantification. *Adipocyte*. 2013; 2(3): 160–164. <http://dx.doi.org/10.4161/adip.24652>. [PubMed: 23991362]
- Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. 2004; 306(5695):457–461. <http://dx.doi.org/10.1126/science.1103160>, PII: 306/5695/457. [PubMed: 15486293]
- Poissonnet CM, Burdi AR, Garn SM. The chronology of adipose tissue appearance and distribution in the human fetus. *Early Human Development*. 1984; 10(1–2):1–11. [PubMed: 6499712]
- Pond CM, Mattacks CA, Calder PC, Evans J. Site-specific properties of human adipose depots homologous to those of other mammals. *Comparative Biochemistry and Physiology Comparative Physiology*. 1993; 104(4):819–824. [PubMed: 8097987]
- Poretzky, L.; Ebooks Corporation. Principles of Diabetes Mellitus. 2010. Retrieved from http://www.msvu.ca:2048/login?url=http://www.msvu.ebilib.com/EBLWeb/patron?target=patron&extendedid=P_511708_0& (pp. 1 online resource (868 pp))
- Pouteau E, Turner S, Aprikian O, Hellerstein M, Moser M, Darimont C, et al. Time course and dynamics of adipose tissue development in obese and lean Zucker rat pups. *International Journal of Obesity*. 2008; 32(4):648–657. <http://dx.doi.org/10.1038/sj.ijo.0803787>. [PubMed: 18087263]
- Reynisdottir S, Ellerfeldt K, Wahrenberg H, Lithell H, Arner P. Multiple lipolysis defects in the insulin resistance (metabolic) syndrome. *The Journal of Clinical Investigation*. 1994; 93(6):2590–2599. <http://dx.doi.org/10.1172/JCI117271>. [PubMed: 8200997]
- Rigamonti A, Brennand K, Lau F, Cowan CA. Rapid cellular turnover in adipose tissue. *PLoS One*. 2011; 6(3):e17637. <http://dx.doi.org/10.1371/journal.pone.0017637>. [PubMed: 21407813]
- Roman AA, Parlee SD, Sinal CJ. Chemerin: A potential endocrine link between obesity and type 2 diabetes. *Endocrine*. 2012; 42(2):243–251. <http://dx.doi.org/10.1007/s12020-012-9698-8>. [PubMed: 22610747]
- Santosa S, Jensen MD. Effects of male hypogonadism on regional adipose tissue fatty acid storage and lipogenic proteins. *PLoS One*. 2012; 7(2):e31473. <http://dx.doi.org/10.1371/journal.pone.0031473>. [PubMed: 22363653]
- Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, et al. Dynamics of fat cell turnover in humans. *Nature*. 2008; 453(7196):783–787. <http://dx.doi.org/10.1038/nature06902>, PII: nature06902. [PubMed: 18454136]
- Sternberg SR. Biomedical Image Processing. *Computer*. 1983; 16(1):22–34. <http://dx.doi.org/10.1109/mc.1983.1654163>.
- Suganami T, Ogawa Y. Adipose tissue macrophages: Their role in adipose tissue remodeling. *Journal of Leukocyte Biology*. 2010; 88(1):33–39. <http://dx.doi.org/10.1189/jlb.0210072>, PII: jlb. 0210072. [PubMed: 20360405]
- Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, et al. Fat tissue, aging, and cellular senescence. *Aging Cell*. 2010; 9(5):667–684. <http://dx.doi.org/10.1111/j.1474-9726.2010.00608.x>. [PubMed: 20701600]
- Tisdale MJ. Biology of cachexia. *Journal of the National Cancer Institute*. 1997; 89(23):1763–1773. [PubMed: 9392617]
- Webster JD, Miller MA, Dusold D, Ramos-Vara J. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. *The Journal of Histochemistry and Cytochemistry*. 2009; 57(8):753–761. <http://dx.doi.org/10.1369/jhc.2009.953877>. [PubMed: 19398606]

Figure 6.1.

Expression of *Adiponectin* increases and *secreted frizzled-related protein 1 (sFRP1)* decreases in epididymal adipose tissue sampled progressively from the testicle. Twelve-week-old C57BL6/J ($N=12$) mice were sacrificed and epididymal adipose tissue harvested. The depot was divided into quarters and orientation to the testicle noted. Total RNA was harvested and analyzed by RT-qPCR. Results indicate an inverse relationship with *adiponectin* expression increasing and *sFRP1* decreasing in samples more distal to the testicle. Two-way ANOVA with Bonferroni post-hoc analysis, $*p<0.05$ when compared to the proximal quarter (area 1).

Figure 6.2.

The distribution of adipocyte sizes and the linear relationship between average adipocyte volume and epididymal WAT weight. Ten-week-old C57BL6/J mice fed a 60% high-fat diet for 6 weeks were sacrificed and epididymal WAT harvested, fixed, sectioned and ~5000 adipocytes per mouse counted using the MetaMorph-based method described herein. The distribution of adipocytes within the adipose depot is calculated using the frequency function in Excel. (A) C57BL6 mice ($N=10$) have a mean adipocyte area of approximately $3600.98 \pm 820 \mu\text{m}^2$ (open circle) and a median of $2970 \mu\text{m}^2$ (black square). (B) The relationship between average adipocyte volume weight of epididymal WAT is linear ($r^2=0.92$).

Figure 6.3.

Adipocytes size and average area are similar when quantified by MetaMorph or ImageJ. Ten-week-old C57BL/6J mice ($N=5$) fed a 60% high-fat diet for 6 weeks were sacrificed and epididymal adipose tissue depots harvested, fixed, sectioned and adipocytes counted using either the MetaMorph or ImageJ method described herein. The frequency distribution of adipocyte sizes is equivalent whether analyzed by MetaMorph or ImageJ. No significant differences were found in either the frequency distribution (A, two-way ANOVA with Bonferroni post-hoc analysis) or average adipocyte area (B, Student's t -test).

Figure 6.4.

Inguinal adipose tissue has smaller adipocytes than epididymal adipose tissue. Ten-week-old C57BL6/J mice ($N=5$) fed a 60% high-fat diet for 6 weeks were sacrificed and epididymal (eWAT) and inguinal (iWAT) adipose tissue depots harvested, fixed, sectioned and adipocytes counted using the MetaMorph method described herein. The distribution of adipocyte areas indicates iWAT has greater numbers of adipocytes with areas below 2000 μm^2 and fewer adipocytes with areas greater than 2000 μm^2 when compared to eWAT (A, B). The area-under-the-curve of this distribution quantifies this elevated number of smaller ($< 2000 \mu\text{m}^2$) and decreased number of larger ($> 2000 \mu\text{m}^2$) adipocyte in iWAT. The difference in adipocyte size is evident in representative H&E stained samples of both iWAT and eWAT (C). Two-way ANOVA with Bonferroni post-hoc analysis, $*p<0.05$ compared to respective adipocyte area increment (A and B).

Figure 6.5.

The frequency of small adipocytes but not the average adipocyte area changes in epididymal adipose tissue located on the testicle. Ten-week-old C57BL6/J mice fed a 60% high-fat diet for 6 weeks were sacrificed and epididymal WAT harvested and divided into five equal sections according to the proximity to the testicle before fixing, sectioning and counting of adipocytes (~1000 per sample) using the MetaMorph method described herein. Whereas the average size of adipocytes did not differ between samples (A), there was a higher frequency of small adipocytes (1000–2000 μm^2) directly adjacent to the testicle (labeled 1). Two-way ANOVA with Bonferroni post-hoc analysis, * $p < 0.05$ compared to samples 2–5.

Figure 6.6.

Counting the area of as few as 100 adipocytes provides an accurate distribution of adipocyte size in WAT. Ten-week-old ($N=10$) C57BL6/J mice fed a 60% high-fat diet for 6 weeks were sacrificed and epididymal WAT harvested, fixed and sectioned. For each individual animal 3, 10, 100, 300, 500 or 1000 adipocytes were randomly counted 10 times and the average adipocyte area calculated. While there was no significant difference in the mean adipocyte area (A) when counting between 3 and 1000 adipocytes, the variance around this mean is significantly greater when counting 3 or 10 adipocytes (B). The distribution of adipocyte areas, however, does not differ whether you count 100, 300, 500 or 1000 adipocytes. Accordingly counting a minimum of 100 adipocytes is sufficient to estimate mean adipocyte size, minimize variance around that mean and provide an accurate estimation of the distribution of adipocytes in WAT. One-way ANOVA with Tukey post hoc analysis, $*p<0.05$ versus 1000 adipocytes counted.

Figure 6.7.

A minimum of five animals are required to detect a ~20–25% difference in mean adipocyte radius. C57BL6/J mice fed a chow diet (22-week old, $N=10$) or 60% high-fat diet (10-week old, $N=10$) for 6 weeks were sacrificed and epididymal WAT, fixed and sectioned. The distribution of adipocyte sizes was used to calculate the mean radius of adipocytes in the epididymal WAT of mice fed standard chow ($26.7 \pm 3.1 \mu\text{m}$) or high-fat diet ($33.8 \pm 3.9 \mu\text{m}$) (A). Average adipocyte volume was calculated and a linear relationship to weight of epididymal WAT observed (B). Based on the calculated variance in the radius of adipocytes from WAT of mice fed a standard chow (C) or high-fat (D) diet, a power equation ($1 - \beta=0.8$, $\alpha=0.05$) indicates that a of five mice would be required to detect a 7 μm or ~20–25% difference between treatments.