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A multi-colour confocal microscopy method for identifying and enumerating macrophage subtypes and adherent cells in the stromal vascular fraction of human adipose.

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ABSTRACT.

This study examines leukocytes present in lymphoedema (LE) adipose tissue (AT) by multi-colour confocal microscopy. LE AT, collected by liposuction surgery, was digested with collagenase to separate adipocytes from other tissue cells, comprising blood and lymphatic endothelial cells, fibroblasts, and all vessel- and tissue-resident leukocytes - the stromal vascular fraction (SVF). SVF cells were activated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, adding Brefeldin-A to prevent cytokine secretion during the final 4 hours. Cells were incubated with CD11b-FITC and CD40-APC (M1 MØ), or CD206-APC (M2 MØ) specific antibodies, fixed, permeabilised, then incubated with either (1) anti-TNF-PE, (2) anti-IL-1β-PE, (3) anti-IL-6-PE, (4) anti-IL-4-PE, (5) anti-TGFβ-PE or (6) isotype-IgG-PE (control), and stained with Hoechst 33342 ~~preserved in permanent mounting media~~ and examined by confocal microscopy. The FITC, PE and APC fluorescence channels were set to achieve minimal cross-channel emission using single-colour controls and voltages set for optimal detection by thresholding on isotype-IgG stained activated cells. Finally, transmission and z-stack images were captured. Cells were analysed as regions of interest (ROI) based on Hoechst-33342 then enumerated as $FITC^+$, $FITC^+APC^+$ or $FITC^+APC^+PE^+$ using an ImageJ script and exported into Excel. This permitted the examination of >9000 SVF cells individually, per LE sample. This method allows for the analysis of a high number of heterogeneous cells defined into any subtype or combination by the investigators' choice of

~~surface and intracellular~~ expression profiles. Fibroblasts, or other cytokine producing cells, can also be analysed by using other antibodies, and the cell count data can be correlated with any clinical or laboratory data.

Key words: Confocal microscopy, fluorescence, macrophage, tissue-resident.

Lit of abbreviations
Allophycocyanin (APC)
fluorescein isothiocyanate (FITC)
Hoechst (H)
macrophage (MØ)
phycoerythrin (PE) and stromal vascular fraction (SVF).

INTRODUCTION.

Human adipose tissue (AT) can be surgically removed by liposuction surgery. Usually, the most common reasons that humans opt for liposuction surgery are for the management and treatment of obesity and/or for cosmetic reasons – a procedure then referred to as liposculpture. These procedures provide an opportunity to examine both and normal and pathologic AT for a better understanding of normal biology and pathological processes at play in various human conditions. A third situation where liposuction is utilised is for the management of advanced primary or cancer-related LE where the lymphoedematous tissue is predominantly AT (Brörson et al., 2006). First pioneered more than 20 years ago in Sweden (Brörson, 2000), and then also in Scotland (Schaverien et al., 2012) and Australia (Boyages et al., 2015), liposuction surgery treatment for advanced cancer-related LE provides proven long-term benefit to LE patients who are then better able to manage their condition provided they continue to comply with compression treatments. Thus, whilst not a cure, liposuction surgery for LE is a hugely beneficial treatment option, increasingly being offered in many countries (Boyages et al., 2015;Brörson, 2015;Schaverien et al., 2012). Although increased fibrosity is also an accepted feature of advanced human LE AT (see International Lymphology Society Lymphoedema position statement (Lymphology, 2020)), the underlying pathology, including the nature of

lipids in human LE adipose, is only recently emerging (Sedger et al., 2016).

One of the issues with any basic histological analysis of LE liposuction tissue (lipoaspirate) is that the nature of the extraction procedure, destroys the tissue architecture to a large extent. Nevertheless, there is increasing evidence of inflammation originating within the AT in a number of human pathologies (Samad et al., 2011;Vandanmagsar et al., 2011), including in cancer-related LE AT (Lin et al., 2012;Sedger et al., 2016). The cellular basis for this inflammation is now emerging and suggestive of a contribution from macrophage (MØ) cells (Sedger, L.M., et al., unpublished). The problem, however, is that upon the first viewing, AT is predominately composed of adipocytes. This is simply because of the sheer size of adipocytes compared to tissue leukocytes. Yet, with higher-power magnification, inflammatory cells can be seen to be present in normal and LE AT, and primarily within 3 locations: (i) junctions between AT adipocytes, (ii) in foci, and particularly (iii) in so called "crown-like" structures (CLS) where a ring of MØ surround and engulf lipids thought to have been derived from dying adipocytes ((Cinti et al., 2005;Haka et al., 2016;Shapiro et al., 2013) and Sedger, L.M., et al., unpublished). MØ in CLS have all the hallmarks of "foam cells" i.e. these MØ cells themselves contain microscopically-refractive lipid-bodies – these are separate and

distinct from normal cytoplasmic lipid droplet structures ((Haka et al., 2016;Shapiro et al., 2013) and Sedger LM., data not shown). However, the presence of these tissue-resident MØ, especially in adipocyte junctions, requires immunohistochemistry and/or use of higher-powered (high magnification) of the AT tissue in order to be visible. Yet, a higher-magnification examination has the effect of either reducing the literal area of AT being examined microscopically or it necessitates considerably longer periods of time for adequate microscope viewing, along with a process for ensuring a strict methodology is employed to prevent re-visualising tissue that have already been viewed/examined. Thus, MØ detection in histological section of adipose is time-consuming and the identification of MØ subtypes by traditional methods is very challenging.

The characteristics of the MØ cells present in human LE AT has not yet reported. The possible processes to examination and characterise the LE AT MØ include the use of multi-colour flow cytometry or microscopy-based methods. Here, we describe a straight-forward 4-colour confocal microscope interrogation and imaging analysis pathway by which MØ can be discriminated into MØ subtypes based on surface expression of CD11b, CD40 (M1 MØ) or CD206 (M2 MØ) and intracellular cytokines TNF, IL-1β, IL-6, TGF-β and IL-4, as used in many previous studies (Butcher and Galkina, 2012;Mia et al., 2014;Shapouri-Moghaddam et al., 2017). This method both identifies and enumerates these cells with high accuracy, without the problem of the MØs being obscured by the much larger adipocytes. Our approach further documents the many different cell types that are present in LE AT stromal vascular fraction (SVF), exactly as described by others (Brestoff, 2017), and provides a highly efficient analysis method to accurately enumerate many different SVF cell types. This includes fibroblasts, blood or lymphatic endothelial cells, to other leukocytes, all present within adipose SVF. This study also provides an essential data handling procedure, necessary to enumerate MØ subtypes by multi-channel fluorescent microscopy, present in 10,000 or so non-adipocyte SVF cells. Importantly, whilst the procedure described here is available to anyone with a basic confocal capable of a simple 4 colour (Hoechst-33342, FITC, PE and APC) detection and access to the free-ware ImageJ software, the method offer flexibility for further adaptations with increased multiplexing of detection parameters. The output data can be tailored the a variety of research goals and enumerated cell types can be correlated to almost any clinical parameter.

METHODS:

Ethics. LE tissue was collected by liposuction surgery from patients at Macquarie University Hospital, with written patient consent and Macquarie University Human Research Ethics Committee approval (protocol # 5201300312). The liposuction surgery was performed by Dr Thomas Lam and Dr Helen Mackie, as described previously (Boyages et al., 2015), according to method established by Prof. H. Brörson (Lund, Sweden) (Brörson, 2000;2016). LE patients were assessed as having advanced LE, based on circumference limb difference (comparing to unaffected limb), chronically elevated L-Dex measurement, and minimal benefit from ongoing conventional treatments of massage and/or bandaging. The liposuction LE AT sample was immediately transported to University of Technology Sydney (UTS) and processed for analysis.

Preparation of stromal vascular fraction (SVF). The non-adipocyte adipose tissue cells were prepared according to the methods described previously (Brestoff, 2017). Briefly, an aliquot of the liposuction tissue samples was incubated at 37 °C in 0.1% collagenase type II (Sigma) prepared in Hanks Balanced Salt Solution (HBSS) containing magnesium and calcium (Life Technology) for 2-3 hrs, with gently rocking. The digested tissue was filtered through a 100 μm gauze (Sefar) into 50 ml tubes and centrifuged at 2000 rpm at 4 °C for 10 mins, to separates adipocytes and non-adipocytic cells – this is known as the stromal vascular fraction (SVF) **(see Graphical Abstract)**. The adipocyte layer was discarded and the cell pellet containing SVF cells as resuspended in $RPMI_{10}$ (RPMI medium supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS), 1% of L-glutamine (Life Technologies), 1% of penicillin/streptomycin (Life Technologies), 1% non-essential amino acids (Life Technologies) and 1% HEPES (4-(2- hydroxyethyl)-1-piperazine-ethanesulfonic acid) (Life Technologies), and centrifuged at 1200 rpm at 4 °C for 5 mins. Erythrocytes in the cell pellet were removed by lysis in ammonium-chloride-potassium (ACK) buffer followed by centrifugation, as above. SVF cells were then cryopreserved in freezing media, comprising 50% heat-inactivated FBS and 10% dimethyl sulfoxide (Sigma) and stored short-term -80° C long-term in liquid nitrogen, before sub-culture and analysis.

Culturing the SVF cells. Circular 1.5 optical glass coverslips or 13mm diameter were dipped in 70% ethanol to sterilise then placed into 24 well tissue cultured plates (Corning). SVF cells were thawed and resuspended in $RPMI_{10}$ and cultured at 37 °C, overnight, to allow them to adhere to the plastic dish. SVF cells, were activated by culturing for 12 hrs in $RPMI_{10}$

containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml of ionomycin (Sigma) to activate the immune cells (Hashimoto et al., 1991). Brefeldin A (Sigma) was added in each well for the final 4 hrs, to prevent cytokine secretion (Fujiwara et al., 1988) and maximise the intracellular cytokine protein detection.

Staining of SVF adherent cells. First, non-specific antibody binding was blocked by culturing the SVF cells in blocking buffer containing 2% heat-inactivated normal goat serum, 2% heat-inactivated normal rat serum and 0.5mg/ml anti-human Ig Fc-receptor blocking antibody (BD Biosciences) for 1 hr at 4 °C. Cells were then incubated in a cocktail of surface CD specific fluorochrome conjugated antibodies: anti-human CD11b-FITC (clone ICRF44), and anti-human CD40-APC (clone 5C3), or anti-human CD11b-FITC (clone ICRF44) and anti-human CD206-APC (clone 19.2) for 1 hr at 4 °C. Additionally, SVF cells were also stained with each antibody singly (to generate single colour controls) or with in isotype-IgG-FITC or isotype IgG-APC control antibodies. Cells were washed three times with $RPMI_{10}$ between staining steps and fixed in 4% paraformaldehyde (PFA)/PBS for 20 mins at 4 °C, then permeabilised in either 0.1% Triton X- 100 (Sigma) or 0.1% Saponin (Sigma) in PBS. Intracellular cytokines were detected in permeabilised cells by incubating in either anti-human TNF-PE (clone MAB11), IL-1β-PE (clone AS10), anti-human IL-6-PE (clone AS12), anti-human IL-4-PE (clone 8D48), anti-human TGF-β-PE (clone TW4-9E7) antibodies, or an isotype IgG-PE control antibody, all from BD Biosciences, in 0.1% saponin/PBS for 45-60 mins at 4 °C. Cell nuclei were detected by adding Hoechst-33342 either in the surface CD-specific antibody staining step, or as a final nuclear staining step. SVF- bound to coverslips were mounted onto clean glass slides (Menzel) using 10µl of glass anti-fade hard-set permanent mounting media.

Confocal microscopy. Images were acquired on a Nikon A1 confocal microscope. This instrument was configured with the following lasers and emission filters to enable detection of Hoechst-33342 (405 nm laser and 425-475 nm filter), FITC (488 nm laser and 500-550 nm detection filter), PE (561nm laser and a TRITC filter 570-620 nm), and APC (640 nm laser with a 663-738 nm detection filter) where each fluorescent channel was excited and detected "in-line" (i.e. channel series) one at a time. An optimal voltage was set by examining the single colour control stained, and isotype control stained SVF cells, in each of the detection channels, using a Nikon 20X NA 0.75 and WD1000 objective, taking care to acquire the image data as z-stacks so as to permit the optimal detection of each fluorochrome i.e. in different focal planes

within the heterogeneous SVF cell sample. For each z-stack, a slice thickness of approximately 1 μm, and approximately 20 steps (a total thickness of around 20 μm) were acquired, starting close to the top and finishing near to the bottom of the cells (or visa versa). Each file was saved as a .nd2 (16 bit) data file. The imaging process was repeated to generate a data set of 10 randomly chosen fields of view for each antibody combination for each liposuction sample processed. Additionally, to confirm that this analysis had correctly detected and assigned each cell type accurately based on its fluorescence phenotype, SVF cells were also imaged with a higher power Nikon 40X NA 1.0 oil objective.

Imaging analysis. Images were viewed with IMARIS (Version 7.0) using the 3D View as a maximum projection image (see Figure 2). Imaging data were analysed via ImageJ (version 1.8.0) or Fiji (version 2.0)(Schindelin et al., 2012). Here, images were duplicated without the transmission channel - to allow the analysis without any background white light. The nd2 files were imported into Fiji and spilt into four fluorescent channels. A Z-Projection image were produced (compressing the z-stack images) in each of the 4 fluorescent channels. The Hoechst channel was duplicated, a threshold for detection was set using the Yen method and the data was converted to mask. Cells on the edge were ignored and water-shedding (on Hoechst) was adjusted to separate any adjacent and touching cell nuclei. Next, thresholds for fluorescent signals were set. Importantly, these threshold levels were set by considering the isotype IgG stained cells, and comparing them to staining on resting versus stimulated cells (see Figure 2). The same threshold was then used for all subsequent analysis and the channels were merged back together.

The Analyze Particle function of ImageJ was used to estimate the total number of cells present in one field of view, with the particle size preference of 20-2000 pixels (ratio 1.6 pixel:∝m) being applied through the Hoechst detection channel - since each Hoechst-stained nucleus represents a single cell. Each cell (or nucleus) was assigned a specifically numbered region of interest (ROI), thus calculating the total number of cells per field of view, and providing a ROI manager list. Next, the average pixel value of fluorescence (in all fluorescent channels) was aligned to each ROI i.e. to each single cell. Finally, the Multi-Measure function was applied, generating a large table of data depicting the cells that were positive for each of the fluorescent channels: $FITC^+$, $FITC^+$ and APC^+, or $FITC^+$, APC^+ and PE^+. Thus, this method allows for the designation of SVF cells as single, double or triple fluorescent positive staining – according to the staining dyes and antibody cocktails used.

Cell subtypes discrimination. To ensure valid representation of the SVF cell composition a total of 10 randomly chosen fields of view were for each patient adipose samples, and each staining cocktail combination, and analysed as described above. The MØ subtypes were:

(1) CD11b-FITC$^+$ and CD40-APC$^+$ = M1 MØ (PE-neg; cytokine-neg)

(2) CD11b-FITC$^+$ and CD206-APC$^+$ = M2 MØ (PE-neg; cytokine-neg)

(3) CD11b-FITC$^+$ CD40-APC$^+$ PE$^+$ = M1 MØ (producing cytokines TNF, IL-1β, or IL-6)

(4) CD11b-FITC$^+$ CD40-APC$^+$ PE$^+$ = Non-classical M1 MØ (producing IL-4 or TGF-β)

(5) CD11b-FITC$^+$ CD206-APC$^+$ PE$^+$ = Classical M2 MØ (producing IL-4 or TGF-β)

(6) CD11b-FITC$^+$ CD206-APC$^+$ PE$^+$ = M2a or 2c MØ (producing TGF-β)

(7) CD11b-FITC$^+$ CD206-APC$^+$ PE$^+$ = M2b MØ (producing TNF, IL-1β, or IL-6)

To efficiently handle the large data sets and calculate the number of each MØ cell subtype, the data (containing 5 channels for 6 MØ cell subtypes and 10 fields of view per staining cocktail) was passed through an automated system of image analysis, by applying the following boutique macro script within ImageJ (shown here below).

```
dir = File.directory;
T=getTitle();
name = getTitle;
dotIndex = indexOf(name, ".");
name = substring(name, 0, dotIndex);
selectWindow(T);

run("Arrange Channels...", "new=1234");
run("Z Project...", "projection=[Max Intensity]");
for (a=0; a<4; a++) {
	Stack.setChannel(a+1);
	//Please uncomment the following four lines for measuring the average pixel
area per ROI
	//run("Threshold...");
	//setAutoThreshold("Yen dark");
	//setOption("BlackBackground", true);
	//run("Convert to Mask", "method=Yen background=Dark calculate black");
}
nNuc=1;
Stack.setChannel(nNuc);
run("Duplicate...","duplicate channels="+nNuc);
setAutoThreshold("Yen dark");
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=20-2000 clear pixel display add");
close();
roiManager("show all");

run("Clear Results")
for (c=0; c<4; c++) {
	Stack.setChannel(c+1);
	for(i=0; i < roiManager("count"); i++){
		roiManager("select",i);
		run("Enlarge...","enlarge=0 pixel");
		getStatistics(area, mean);
		wait(1);
		setResult("Channel_"+c+1+"_AveragePixelValue", i, mean);
	}
```

```
}
updateResults();
saveAs("results",  dir + name + ".csv");
```

Data handling code. To efficiently process the .csv file data, the output data was imported into a Microsoft Excel file for further processing and results graphing. First, a threshold value across each channel was determined (based on isotype-IgG stained cells - refer to Figure 2) to distinguish positively stained cells from cells not expressing the antigens being detected; for example, in this study Hoechst was set to 240, and PE was set to 36. Second, formulas were used to enumerate each cell type (single, double and triple fluorescent positive cells) within each tissue sample; Where channel B is Hoechst, channel C is PE, channel D is FITC and channel E is APC. This process was repeated for the data obtained from each field of view and combined. Together, these processes enabled easy analysis of 4 (or more) channels of microscopic data, from up to or <8,000 cells, per liposuction tissue sample. The output data can also then be graphed in Excel (Microsoft Office) or via another software program, and the output data analysed statistically, such as with Prism (GraphPad) software or any other statistical analysis software.

```
1.PE only: =COUNTIFS(B:B, ">=240", C:C,"=0", D:D,">=36", E:E, "=0")
2.FITC only: =COUNTIFS(B:B, ">=240", C:C,">=37", D:D,"=0", E:E, "=0")
3.APC only: =COUNTIFS(B:B, ">=240", C:C,"=0", D:D,"=0", E:E, ">=17")
4.FITC + PE: =COUNTIFS(B:B, ">=240", C:C,">=37", D:D,">=36", E:E, "=0")
5.APC + PE: =COUNTIFS(B:B, ">=240", C:C,"=0", D:D,">=36", E:E, ">=17")
6.FITC + APC: =COUNTIFS(B:B, ">=240", C:C,">=37", D:D,"=0", E:E, ">=17")
7.FITC + PE: =COUNTIFS(B:B, ">=240", C:C,">=37", D:D,">=36", E:E, ">=17")
8.Total number of cells: =COUNTIF(B:B, ">=240")
```

RESULTS:

To examine SVF cells using multi-colour confocal microscopy, first one must carefully choose a combination of dyes and fluorochrome-conjugated antibodies that could be detected with the available instrument laser and detection filter combination. Using a Spectrum viewer program, a 4-colour system was designed to detect and identify M1 and M2 MØ cells in SVF samples: nuclear staining with Hoechst-33342, anti-human CD11b-FITC as a macrophage marker – together with anti-CD40-APC (M1 MØ) or anti-CD206-APC (M2 MØ). (For review see (Butcher and Galkina, 2012;Shapouri-Moghaddam et al., 2017)). Since the human tissue sample, lymphoedema adipose tissue (liposuction sample), has not been extensively examined previously, this system was required to simultaneously detect unusual MØ phenotypes and characterise the sub-types. The cytokine staining was therefore performed in parallel: TNF, IL-1β, IL-6, IL-4 TGF-β and compared to an isotype control IgG-PE antibody. This combination of FITC, APC, PE fluorochromes and Hoechst-33342 was

predicted and confirmed be detectable without cross-channel bleed-through via the assessment of single colour controls in all detection channels **(Supplementary Fig 1)**.

Although this four-colour detection of nuclei, surface CD antigens and intracellular Brefeldin-A-blocked Golgi cytokine protein was predicted to be possible, this method must deal with the fact that the positive fluorescent signals are present in different sub-cellular locations, and thus, in different focal planes of SVF cell cultured monolayers. It was therefore necessary to examine the diverse myeloid and epithelial adherent cell types via an extensive z-stack approach so as to ensure that all fluorescent signals would be collected into the same Z-Projection image, and not yield, instead, a falsely cytokine negative (PE negative) single selected focal plane **(Figure 1)**. Hence sufficient slices must be sampled so as to cover as close as possible to the entire cell thickness of all cell types, yielding a high likelihood of detection a positive fluorescent for both surface antigens and intracellular cytokines **(Figure 1C)**.

Figure 1: Data acquisition design. (A) Human AT SVF samples contain various cells of different sizes and morphology; cartoons from BioRender.com. **(B)** A single "slice" image acquisition is unable to simultaneously detect both surface and intracellular cytokines in all cells present within the SVF sample. **(C)** Three-dimensional images creased by capturing z-stack slices through the SVF sample, with surface and intracellular staining data from all cell types. **(Colour figure – 2 column widths)**

Similarly, the detection of a positive fluorescent signal required the delineation of both auto-fluorescence and non-specific binding, that is, the background levels of the staining. For this reason a threshold of fluorescence intensity was determined for FITC, APC and PE using a panel of isotype IgG-fluorescently conjugated antibodies compared to the selected detection antibodies anti-CD11b-FITC, CD20-APC, IL-4-PE and Hoechst-3342.This approach accounts for different expression levels of the detection antigens, in resting and activated cells **(Figure 2)**.

(Colour – 2 column width) Figure 2: Data acquisition for positive threshold setting. (A) Staining with a panel of isotype IgG-matched fluorescently-conjugated antibodies, or **(B)** CD- and cytokine specific antibody staining on resting and PMA plus ionomycin activated SVF

cells. Data is fluorescent intensity plots of fluorescent signals detected; y-axis height (scale) is auto-generated and cannot be set manually. Data shown is representative of analysis of 3 LE AT SVF adherence cell cultures. **(Colour figure – 2 column widths)**

Next, the detection of cytokines in phorbol-myristate-acetate (PMA) and ionononomycin-activated SVF cells was used to optimise the method of permeabilization and preservation of intracellular cytokine antigens. Although both saponin and triton-X100 were able to facilitate the intracellular detection of IL-4 accumulation, greater fluorescence was detected using 0.1% saponin **(Supplementary Figure 2)**. Thus, saponin was chosen as the permeabilising agent for all subsequent staining.

Having optimised the staining and confocal microscopy detection conditions, SVF were stained with the full panel of antibodies and Hoechst-33342 and 10 randomly chosen fields of view were imaged using up to 20 z-stacks for the image capture. As noted above, the staining cocktails focused on detection of M1 MØ with the combination of CD11b-FITC^{+} CD40-APC+ and each cytokine-PE intracellular antibody, plus an isotype IgG-PE stained control antibody. The same approach detected M2 MØ with antibodies to CD11b-FITC and CD40-APC plus cytokine-PE conjugated antibodies. These images were easily viewed with Imaris software showing the individual cell detection in the context of the SVF cultures **(Figure 3)**. Here, it appeared that this methodology had detected CD11b-FITC and CD40-APC (M1 MØ) secreting either TNF, IL-1β, IL-6, or non-classical M1 MØ cytokines TGF-β or IL-4 **(Figure 3A)**, and the CD11b-FITC and CD206-APC (M2 MØ) secreting either IL-4 or TGF-β. However, we also detected CD11b-FITC and CD206-APC (M2 MØ) secreting TNF, IL-1β, or IL-6 typical of M2b MØs (Figure 3B), consistent with the fact that there are actually at least 3 categories of M2 MØ (Butcher and Galkina, 2012;Shapouri-Moghaddam et al., 2017).

Figure 3: Confocal Microscopy detection of M1 and M2 MØ. SVF cells were examined by confocal microscopy, capturing 3-dimentional z-stack data. **(A)** SVF stained with antibodies to CD11b-FITC and CD40-APC to detect M1 MØ, or **(B)** CD11b-FITC and CD206-APC to detect M2 MØ. Both M1 and M2 MØ were further sub-typed according to their production of intracellular cytokines, compared to isotype-IgG PE antibody, after 12 hours of activation with PMA and ionomycin. Brefeldin A was added in the last 4 hours of activation. Data shown is representative of analysis of 10 randomly chosen fields of view of each combination, from adherent SVF cell cultures from each liposuction sample. **(Colour figure – 2 column widths).**

Next, the image data were thoroughly analysed and an automated system established to enumerate each of the different fluorescently identified cell types. As stated above, the first step was the collection of 3-D-confocal images (step 1)(Figure 4). Next, the 3D-confocal images were converted into a single Z-projection plane with ImageJ (Step 2). The fluorescent channels were then split (step 3), the thresholds for each channel were set, and the data made binary (step 4). Individual cells were separated from clumps by water-shedding on Hoechst 33342 (step 5), and finally the data in each channel was merged together (Step 6). Each individual cell was designated as a region of interest (ROI) (step7) thereby also generating a ROI data list where each cell, now defined as a Hoechst-positive ROI, was also scored for fluorescence area intensity for FITC, APC and PE (Figure 4).

Figure 4: Image data processing. Three-dimensional z-stack data was processed in image J. Multi-colour image data acquired in Nikon A1 confocal microscopy via NIS Elements (step 1), data processed as z-project in Image J (step 2), fluorescent channels were separated (step 3), data in each channel was made binary for separate independent channel processing and threshold setting (see supplementary Figure 3)(step 4), Individuals cells were separated by water-shedding on Hoechst-stained cell images (step 5), fluorescent channel data was re-merged (step 6), and a cell count was determined by setting a region of interest (ROI) count (step 7), then, finally, the ROI list data was extracted into xls from the analysis (step 8). Since 10 random fields-of-view of imaging data was collected from each SVF, the imaging data process was further automated by establishing a processing instruction computer code (see methods for automation coding text). The process was followed for each combination of staining, for all patient samples analyzed. **(Colour figure – 2 column widths)**

To confirm that this method had indeed correctly counted a Hoechst-stained cell as co-fluorescent (FITC plus APC and/or PE) the ROI was examined by manually adding successive 1 pixel wider diameters (1 pixel, 2 pixels, through to 5 pixels) and 5 fields were re-counted manually i.e. comparing the ROI auto-calculation against a manual modified count. In each situation where the pixel size was increased the cell counts statistically significantly increased, indicating that the auto-calculated counts were in fact an accurate cell count and the extra pixels were not required but had in fact also detected adjacent fluorescent cells (data not shown). Finally, to provide additional confidence in the method's capacity to detect a MØ cell subtypes, high-power images were also obtained **(Figure 5)**. Shown is a single chosen low magnification power field of view where single CD11b-FITC positive cells (green), single CD40-APC$^+$ cells (purple) and single IL-4-PE$^+$ cells, as well as well as CD11b-FITC$^+$ and CD40-APC$^+$ (M1 MØ) or CD11b$^+$ and PE-cytokine producing cells (i.e. negative for CD40 or CD206) are evident. Triple positive CD11b-FITC$^+$ CD40-APC$^+$ and PE$^+$ cytokine producing TNF$^+$ cells etc, were also present in this field of view (Figure 5A). Nevertheless, to confirm the methodology

had indeed identified different M1 and M2 MØ cell types, an additional higher-powered imaging was performed. Examples of TNF and IL-1β producing PE^+ CD11b-$FITC^+$ CD40-APC^+ M1 MØ, as well as IL-4 producing CD11b-$FITC^+$ CD206-APC^+ M2 MØ are shown (without 3D z-stacks) (Figure 5B).

Figure 5: Confirmation of diverse cell types. (A). The imaging strategy of 4-colour staining can detect several MØ cell types, as well as non-myeloid lineage cell types, simply on the basis of FITC, APC, and PE positive or negative staining results, with each cell detected by Hoechst nuclear staining. **(B)** Confirmation of each individual cell types was determined by higher-magnification images (40X objective). Shown (left to right) are M1 MØ $CD11b^-FITC^+$ CD40-APC^+ TNF-PE^+, M1 MØ $CD11b^-FITC^+$ IL-1β-PE^+ and M2 MØ $CD11b^-FITC^+$ CD206-APC^+ IL-4-PE^+ as individual cell examples. Panel A is representative of all fields of view (10 random fields chosen), dependent on the reagent combination. Panel B is representative of 3-5 high power magnification images. **(Colour figure– 1 column width)**

Considering all of the images together it was clear that LE SVF CD11b-$FITC^+$ CD40-APC^+ M1 MØ are usually producing cytokines TNF, IL-1β, or IL-6, yet other CD11b-$FITC^+$ CD40-APC^+ SVF cells were unexpectedly detected expressing cytokines TGF-β or IL-4 (Figure 4). The LE SVF $CD11b^+$ $CD206^+$ M2 MØ were producing a range of cytokines as well. This was not surprising since it is consistent with fact that M2 MØ are often further categorised into subsets. For example, M2a MØ typically producing IL-10, TGF-β, and chemokines CCL17, CC18, CCL22; M2b MØ producing TNF, IL-1b, IL-6, IL-10 and CCL1; M2c with IL-10, TGF-β, CCL16, CCL18 and CXC13, M2d MØ expressing IL-10 and VEGF, these subtypes being evident in different mouse or human tissues in range of pathological conditions (Shapouri-Moghaddam et al., 2017). Thus, $CD1b^+$ CD206 M2 MØ expressing TNF, IL-1β, or IL-6 detected in our analysis are consistent with chronic nature of LE and their known roles in anti-inflammatory tissue remodelling (Shapouri-Moghaddam et al., 2017). Indeed, because the imaging had been performed with low power 20X objectives, and 10 randomly chosen fields of view, this method has minimal unconscious bias (apart from investigator choice of the target organelles or antigens being detected), and enables the detection of diverse MØ subtypes based on a simple paradigm of CD11b with CD40 or CD206 and the cytokine expression. Thus, this method can detect all MØ subtypes, of various frequencies, from amongst literally thousands of SFV cells (10 fields of view can produce data from up to 10,000 SVF cells) per adipose tissue biopsy sample. The final step of employing this method is, henceforth, one that enables ease of data handling. For this reason the output .csv file was imported into an Excel file, and a formula was written and enable the auto-processing of the complex image analysis data files, i.e. to enumerate all of the possible individual cell types identified in the data (see methods section for the formula). The data can then be graphed for visual presentation and statistically analysed to compare various cells both within a single sample and across different patient groups (e.g. either adipose tissue samples from different

patient cohorts, or experimental groups). In the time we have utilised this method, several LE AT samples have been examined, each with different number of SVF cells per biopsy sample (data not shown), we therefore recommend normalising to the least number of SVF cells in a given patient tissue sample. In our experience examining LE AT this is usually best converted a number per 3000 SVF cells, per LE patient sample, but this might also be guided by lowest number of SVF cells imaged which will be depend on the number of fields of view captured and analysed by this method.

DISCUSSION.

Adipose tissue (AT) is frequently extracted by liposuction surgery in clinical conditions of obesity or via so called liposculpture for cosmetic reasons. In either situation the suction-based extraction of tissue renders an histology analysis somewhat problematic i.e. limited to remaining "intact" AT. Additionally, the AT sample is by its very nature complicated by the abundance and sheer size of the adipocytes relative to the other tissue resident cells. For example, AT obviously contains numerous non-adipocyte cells, including AT fibroblasts that are non-evident in H&E sections unless viewed by immunohistochemistry, or viewed by electron microscopy, and there is a supporting connective tissue structure of collagen (Ross M.H. and Romrell, 1995). Also present are blood vessels and lymphatic vessels comprised of specialised endothelial cells ($CD34^+$ blood vessel endothelial cells and podoplanin expressing lymphatic endothelium) together with their adjacent helpers – the vessel pericytes. Intra-vessel leukocytes and extravasated inflammatory leukocytes are also present in AT. Thus, the examination of AT leukocytes is compromised by disruption of tissue architecture due to liposuction vacuum-based tissue extraction, and the dominating feature – the much larger adipocytes. Together, these factors arguably renders conventional histology a less preferred method for detection and enumeration of inflammatory cells that are present in normal or pathological AT when collected by liposuction surgery. Possible alternative approaches to examine the tissue leukocytes include confocal microscopy or flow cytometry analysis of the non-adipocyte fraction collectively known as the stromal vascular fraction (SVF). One problem that arises, however, is that stimulation of blood, lymphatic or tissue-resident macrophages renders them strongly adherent, meaning that flow cytometry-based detection of cytokines in PMA-activated SVF macrophage cells is difficult as it requires cells liberation from the tissue culture vessel after activation/adherence, prior to analysis. Whilst this is theoretically possible, issues such as over-trypsinization can impact on surface antigen detection or conversely non-liberated cells that remain adherent are lost from the cytometry-based analysis. Instead, we describe here, a confocal microscopy-based method for the detection and accurate enumeration of SVF adherent cells, focusing in this case on $CD11b^+$ cells, predominately MØ subtypes based in part on cytokine production, by a simple 4-colour

fluorescent staining regime, and the subsequent automated data processing of multiple fields of view, that together, enable the analysis of several thousands of SVF cells, yielding a reliable enumeration of multiple diverse MØ cell types. Thus, whilst both the generation of SVF from adipose, and confocal microscopy are, themselves, independently well-established techniques, the method described here sufficiently embraces the highly heterogeneous nature of SVF cells for microscopic analyses (otherwise arguably more easily analysed by flow cytometry), as well as the inherent issues of sensitivity for the detection of varying levels of intracellular cytokines, including the capacity of identifying low abundance cytokine positive cells with high confidence.

Moreover, the purification of non-adipocytes from mammalian AT by collagenase digestion yields an extremely heterogeneous cellular SVF sample. Thus, virtually all non-adipocyte cells normally present in AT SVF can be detected, examined and quantitated. Particular cell types can also be purified, cultured, and/or analysed metabolically or biochemically, either individually or together with other selected SVF cell types. Here, we have chosen to use a simple 4-colour confocal microscopy method to dissect the $CD11b^+$ cell types present within whole (non-cell specific) SVF samples. While it is acknowledged that the preparation of SVF requires collagenase enzymatic digestion, the overnight culture and recovery period permits the examination of surface proteins without significant loss of known surface antigenicity (i.e. antibody-based identification) – as demonstrated here and previously (Brestoff, 2017). By coupling the CD11b expression with other surface antigens, in this case either CD40 (M1 MØ) or CD206 (M2 MØ), we have begun to delineate M1 and M2 MØ subtypes in LE SVF. Traditionally, $CD11b^+$ $CD40^+$ M1 MØ express TNF, IL-1β and/or IL-6 cytokines (Sica and Mantovani, 2012), however, we also detected non-classical M1 MØ expressing TGF-β and IL-4. The function of these cells is unknown. Similarly, while $CD11b^+$ $CD206^+$ identifies M2 MØ that typically express IL-4 and/or IL-10 (Sica and Mantovani, 2012) this method identified more M2 MØ expressing TNF, IL-1β, IL-6, or TGF-β, than those expressing IL-4 (enumeration data not shown). Rather than considering this an invalidation the CD11b and CD40/CD206 plus cytokine sub-type identification paradigm for the delineation of M1 and M2 MØ, it is important to consider the unique pathology of LE AT that is currently still largely uncharacterised. TNF, IL-1β and IL-6 producing M2 $CD11b^+CD40^+$ are likely to be M2b MØ that are known for their role in immune regulation (Shapouri-Moghaddam et al., 2017). Furthermore, the production of TGF-β may indicate that M2c MØ are present in LE AT and involved in phagocytosis of apoptotic cells (Shapouri-Moghaddam et al., 2017) but we also detected $CD11b^-CD40^-$ and $CD11b^-$CD206- cells producing TGF-β that we predict are most likely to be fibroblasts. Thus, the data generated from this microscopic analysis of LE AT SVF

is capable of providing insights into the importance of TGF-β and the mechanisms of fibrosis in LE AT pathology (Wang et al., 2016). In any case this paper is focused on describing a detailed microscopy methodology – and not the pathology of human LE adipose per se – precluding further discussion on the biological functions of various adipose tissue MØ cell types detected by this method at this point in time. One must also acknowledge that this study we examined SVF cells after 12 hours of stimulation via PMA and ionomycin (with Brefeldin A added in the last 4 hours) meaning that different cytokine expression patterns are expected if the stimulation conditions are shortened or extended before the addition of Brefeldin A. Any comparison of our data to other publications describing AT MØ needs to carefully consider the culture similarities or differences, and the type of activation stimuli.

By using microscopes fitted with additional lasers the detection of intracellular cytokines can easily be multiplexed. In this example we have used a 4-colour staining panel and can compare the number of $CD11b^{+}CD40^{+}$ M1 or $CD11b^{+}CD206^{+}$ M2 MØ and their cytokine expression. The capacity of newer confocal microscopes to deliver greater than 4- fluorescent channels creates additional opportunities to further refine the methodology with greater cell specific identification via other parameters, be they surface or intracellular parameters. With such capacity one might ideally include detection of arginase-1, IL-10, and/or other carefully selected chemokines to facilitate MØ subtype identification. The only caveat is the continued need to confirm baseline fluorescent detection by setting thresholds for defining fluorescence positivity - exactly as we have done here, and the use of single colour controls staining - to find staining protocols with limited or no bleed-through into adjacent detection channels. We have already adapted the technique to include an additional fluorescence channel to detect AT fibroblasts using a two-step surface staining method with the TE7 human fibroblast-specific antibody (Goodpaster et al., 2008) (see Table 1, and data not shown). One could also easily simultaneously embrace the use of CD34- and podoplanin- specific antibodies to detection and investigate blood and lymphatic endothelial cells present in SVF (see Table 1). The capacity to add additional markers is also dependent on the availability of other unique and validated monoclonal antibodies, the careful choice of antibody-conjugated fluorophores. The use of tuneable lasers in microscopy further broadens fluorochrome choices and enables the potential detection of naturally occurring auto-fluorescence as a metabolic signature. In this respect there is also great potential to embrace boutique fluorescent-traceable analogues of cellular metabolites, such as for the detection of key biochemical metabolic signatures within certain SVF cell types. Importantly, however, we have clearly demonstrated the value of the dove-tailing a coded data analysis to facilitate data handling of thousands of individual cells and their fluorescent signatures. This data management aspect becomes increasingly critical as more parameters are included in multi-colour confocal microscopy data analysis, and it

simultaneously enables high numbers of cells to be identified with high certainty. Taken together, we have described in detail a system of confocal imaging and data analysis, including the critical points for ensuring an accurate cell-type specific identification, for the identification and enumeration of AT SVF cell types.

Table 1: Cell type identification system based on CD11b, CD40 (or CD206) and cytokine expression.

(A) SVF:Myeloid Cell Types: MØ Sub-Types	(CD11c^{+})	CD11b^{+}	CD40^{+}	CD206^{+}	TNF^{+}	IL-1β^{+}	IL-6^{+}	IL-4+	TGF-β^{+}
Macrophage sub-types									
Classical M1 MØ									
Classical M1 MØ									
Classical M1 MØ									
Non-Classical M1 MØ									
Non-Classical M1 MØ									
Classical M2 MØ									
Classical M2 MØ									
M2a MØ									
M2b MØ									
M2c M2 MØ									
(B) SVF: Non-Myeloid Cell Types: Vessel & Connective Tissue Cells	TE7^{+}	CD11b^{+}	CD34^{+}	Podoplanin	TNF^{+}	IL-1β^{+}	IL-6^{+}	IL-4^{+}	TGFβ^{+}
Fibroblast									
Blood endothelial cell (BEC)									
Lymphatic endothelial cell (LEC)									

The following are the supplementary data related to this article.

Supplementary Figure 1: Spectral viewer & Single colour controls. (A) A plan for 4-colour detection of MØ subtypes was devised based on specific monoclonal antibodies conjugated to FITC, APC and PE, and the possible laser and detection filters available on the Nikon A1 confocal microscope. **(B)** The planned staining utilising Hoechst-33342 for detection of all cells via nuclei, surface FITC CD11b-expression on myeloid cells, surface CD40-APC or CD206- APC for M1 and M2 MØ subsets, and intracellular cytokine (-PE conjugated antibodies). **(C)** A set of single colour controls and unstained cells were imaged in all channels to established voltage gain settings, ensuring clear detection of each fluorochrome without significant bleed though into other fluorescent channels. **(Colour Figure – 2 column widths)**

Supplementary Figure 2: Permeabilization optimisation for intracellular cytokine detection. (A) SVF cells were stained with a panel of antibodies specific to CD11b-FITC and CD206-APC, them fixed in 4% PFA/PBS, and permeabilized with either 0.1% saponin/PBS or 0.1% triton-X100 detergent /PBS and incubated with intracellular IL-4 cytokine-PE conjugated antibodies. Cells were washed and images in FITC, APC and PE channels to determine optimal detection of CD and intracellular antigens. Data shown is representative of analysis of 3 LE AT SVF adherence cell cultures. **(Colour Figure – 2 column widths)**

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COMPETING INTEREST STATEMENT.
All authors state they have no conflicts of interest to report.

AUTHOR ATTRIBUTION STATEMENT:

AD and LMS conceived and designed experiments.

AD and LMS processed liposuction tissues.

AD, MJ, and LMS performed experiments.

AD, ZN, MJ and LMS contributed to data analysis.

AD and LMS wrote the paper and prepared data figures.

Supplemental Figure 2. Data acquisition – Permeabilization optimization.

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Graphical Abstract: Scheme for Adipose Tissue processing and SVF Macrophage (MØ) Cell Imaging.

(A) Sample preparation. LL patient liposuction surgery lipoaspirate samples were collected directly from the surgery theatre, transported to the laboratory, and aliquots were digested with 0.1% collagenase for 2-3 hours, gauze-filtered (100μm), and centrifuged to separate adipocytes from non-adipocytes. Erythrocyte red blood cells (RBCs) were removed by a lysis to yield the Stromal Vascular Fraction (SVF) cells. Finally, SVF cells were cryopreserved at -80 °C (for short-term) and/or liquid nitrogen (for long-term) storage. **(B) SVF Adherent cells.** Adherent cells present in SVF are examinable by confocal microscopy after culture on microscope coverslips in tissue culture dishes. Other non-adherent immune cell types are also present in the SVF samples and can be analysed by other methods (not shown). **(C) Confocal microscopy.** Imaging and a subsequent data analysis pathway was established to identify and enumerate specific cell types, in this instance with the focus on M1 and M2 MØ subtypes. Cell cartoons shown in B and C were generated from BioRender.com. **(Colour Figure – 2 column widths).**

HIGHLIGHTS.

Supplemental Figure 2. Data acquisition – Permeabilization optimization.

- Liposu
- Adipose histology is limited to intact tissue and dominated by adipocytes
- Stromal vascular fraction leukocytes contribute inflammation via diverse cell types
- Confocal imaging offers a flexible platform for identification of multiple cells
- Careful imaging data processing is critically important for accurate cell counts
- Coded analysis enables high-throughput identification of non-adipocytes in adipose