International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Application of Etaluma Immunofluorescence Microscope for Saliva Protein Analysis

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Abstract: ***This study investigates the application of the Etaluma Immunofluorescence Microscope in analyzing growth factors and cytokines in saliva. Saliva samples, which offer a non-invasive collection method, were examined for their protein content using immunofluorescent markers. The research outlines the benefits of this microscope for small labs and practitioners, emphasizing the ease of use and economical aspects. This methodology provides a promising option for saliva-based analysis of proteins relevant to stem cell therapy and other therapeutic interventions.***

Keywords: saliva protein analysis, immunofluorescence microscope, cytokines, growth factors, saliva diagnostics, Etaluma Immunofluorescence Microscope, Ouchterlony test, modified Ouchterlony double immunodiffusion

1. Introduction

Many naturopathic physicians and other alternative medicine providers have both an interest and a need for economical saliva protein testing. Saliva testing is preferred in many alternative medicine protocols because serial testing over extended periods, as well as before and after testing, can be achieved with almost no patient discomfort. Without the need for numerous blood tests and needle pricks, patients are more likely to accept alternative medicine therapies.

With the increased focus on the role of growth factors and cytokines, both as players in pathological conditions, and their availability in saliva, the need for economical saliva testing has arrived.

With regard to the advancing field of stem cell therapeutics, Drake and Hicks were first to publish a Table demonstrating that the available growth factors and cytokines were nearly identical for Adipose Tissue, Stromal Vascular Fraction (SVF), Amniotic Fluid, and Platelet Rich Plasma (PRP) [1]. The Table is shown here, with permission of the authors, references omitted [See Table Comparing Major Factors/Cytokines Below References]. Those authors went on to propose that due to the near identity of growth factors in PRP when compared to the typical SVF used in stem cell therapeutics, PRP alone could be used for therapy obviating the need for any mini-liposuction normally used in every SVF procedure.

Shortly thereafter, colleague Rodney Villalobos, also writing for the Panama College of Cell Science, reported nearly identical growth factors and cytokines were present in saliva as well, thereby opening the door to the usefulness of saliva testing for these molecules [2].

Table Comparing Major Factors/Cytokines
(Copyright Rodney V. Villalobos January 1, 2024)
References Omitted, Used with Permission

Growth Factors/Cytokines	PRP	Saliva
CTGF Connective Tissue Growth Factor	✓	✓
ECGF Endothelial Cell Growth Factor	✓	
bFGF Basic Fibroblast Growth Factor	✓	✓
TGF β1 Transforming Growth Factor Beta 1	✓	✓
VEGF Vascular Endothelial Growth Factor	✓	✓
IGF 1&2 Insulin-like Growth Factors	✓	✓
PD-EGF Platelet Derived Epidermal Growth Factor	✓	
PDGF-α and β Platelet Derived Growth Factor	✓	✓
FGF-2 Fibroblast Growth Factor 2	✓	✓
IL-6 Interleukin 6	✓	✓
IL-8 Interleukin 8	✓	✓
IL-1β Interleukin 1β	✓	✓
IL-10 Interleukin 10	✓	✓
IL-12p70 Interleukin 12p70	✓	✓
TNF-α Tumor Necrosis Factor Alpha	✓	✓
NGF Nerve Growth Factor	✓	✓
HGF Hepatocyte Growth Factor	✓	✓
EGF Epidermal Growth Factor	✓	✓
GCSF Granulocyte-Colony Stimulating Factor		
M-CSF Macrophage Colony Stimulating Factor		✓
FGF4,7,19,21 Fibroblast Growth Factors 4,7,19,21		
SCF Stem Cell Factor		✓
Fibrinogen	✓	✓
PF5 Platelet Factor 5	✓	
P-selectin	✓	✓
α-granules, dense granules, and lysosomes	✓	✓
MCP 1 Monocyte Chemoattractant Protein 1		✓
GM-CSF Granulocyte Macrophage Colony Stimulating Factor		✓
IL-2 Interleukin 2		✓
IL-4 Interleukin 4		✓
SDF-1 Stromal Cell Derived Factor 1		✓

What has been lacking in stem cell therapeutics in general, and more particularly alternative medicine, is the lack of economical and available patient testing of growth factors and cytokines. Although 25 substrates available for testing in saliva and 22 substrates for testing in PRP, only a very small number of these molecules is even offered for testing at clinical blood testing labs.

Currently, the assessment of growth factors and cytokines can be determined by high-performance liquid chromatography, mass spectrometry, or protein array tests, all out of reach except by only the most well-funded of research institutions. Even Enzyme-Linked Immunosorbent Assay (ELISA) and multiplex immunoassays such as Luminex or Bio-Plex are equally out of reach for single patient work-up by physicians and alternative medicine practitioners. Neither any patient or practitioner can test for these substances in blood or saliva for less than thousands of dollars, making such testing totally unavailable due to cost.

The purpose of this report is to present a review of the **Etaluma Immunofluorescence Microscope** that can, in tandem with simple gel-diffusion immunofluorescent techniques, yield a low cost, yet quantitative measuring of growth factors and cytokines in saliva. This remarkable unit and software can be adapted to both gel electrophoresis investigations, as well as even simpler immunodiffusion techniques to yield both a cost effective and relatively easy investigation of saliva proteins. Both the economical cost and its utility by the individual practitioner seeking to measure single patient results makes the **Etaluma scopes and associated software** particularly worthy of consideration by small labs and even practitioners interested in "in-house" saliva testing.

2. Materials and Methods

The experimental design involves challenging saliva samples with antibodies conjugated to immunofluorescent markers. In a gel diffusion or electrophoresis format, antigen-antibody complexes form precipitin bands, which are analyzed using software-equipped immunofluorescent microscopy

Hardware. The author conducted a website review and a literature review of the many immunofluorescence microscopes currently being marketed for "immunofluorescence" research studies. The inquiry focused on both the hardware supplied, as well as associated lenses and software. Software is essential to the overall result obtained because the software is necessary to convert the observed fluorescent signal into a quantitative result. It is not appropriate to note all the many manufactures and products reviewed, except to report that the investigation was exhaustive. Most immunofluorescence scopes are used for cell-based analysis of proteins and molecules inside cells or associated with cell membranes and were more complex than needed or totally inappropriate for simple gel-based studies. Many of these were set up primarily for 96 well plates involved in cell culture studies, which require different unneeded capabilities. The investigation of gel-based immunodiffusion via immunofluorescent microscopes, so prevalent in the 1970's in the study of immune complexes, seems to have been largely left behind in the advance toward ever more complicated, and extremely expensive, hardware and software. The new confocal immunofluorescent microscopes start at around $250,000. For university and research institutes there is an abundance of very fine, though expensive, equipment to be sure.

Software. In addition to hardware, the ease of use of the software for reading gel diffusion bands is critical to the assay results. While many sellers were quick to respond with prices for the microscopes, most had rather uninformed staff and complex messaging systems when it came to discussing the associated software and/or obtaining demonstrations or even illustrations as to how to use it. Most if not all current software seems needing to be modified from reading well plates to reading bands of fluorescence in gels. In addition, it was disappointing to learn how many manufacturers relied on ImageJ software for interpreting fluorescent signals. The author found **ImageJ software** to be cumbersome and bulky and not recommended for any individual practitioner or small lab due to extensive learning curve required. Yet, most sellers just referred the author to ImageJ software for use with their scopes with no helpful guidance as to how it is adapted to their specific hardware. Understandably, whenever a new technique as here is being devised, it many times does not fit within the parameters of the existing and popular suppliers. **One stand-out in software is the product engineered by Silk Scientific**, of Provo, Utah [SilkScientfic.com]. Dr. Jeffrey Silk has a very nice, easy to use gel reading product in his UN-SCAN-IT software which the author found both easy to use and economical as well. Annual License is advertised at $129 USD for Windows or Mac; $445 USD permanent license for the UN-SCAN-IT Gel Analysis Software. Moreover, Dr. Silk is happy to assist with modification and use support. The author would have selected this product but for the very nice associated software offered by Etaluma.

Digitometers: In addition to fluorescent microscopes, there are quite a few digitometers for reading gel scans. Most of these are set-up to take a scan of an electrophoresis gel sheet, and convert the scan to quantitative results. **The majority rivaled the costs of the Etaluma package without the fine microscopic sensitivity** required for detecting tiny fluorescent activity of low concentration proteins. Because growth factors are in very low quantities in saliva, the highest detectible sensitivity is required for saliva analysis in the author's opinion. The iPhone app for reading gels was very easy to use but lacked sensitivity for low fluorescence, as was the case for the other low-cost densitometers. Thus, digitometers were ruled out.

Etaluma: The author settled on the Etaluma Immunofluorescent Microscope with Windows or MAC software [Etaluma Inc., 3129 Tiger Run Court, Carlsbad, CA 92010]. The website is Here: https://etaluma.com Etaluma offers two models as of this writing: LS820; and the more automated LS850. The following review is based on the LS820 with "Blue, Green, and Red Fluorescence with Autofocus and Z-Stacking for Time Lapse

Automation". While Etaluma is also moving toward a primary use in live cell imaging and cell growth studies, yet, their hardware and software is nonetheless easily adaptable to reading immunofluorescent gel bands. As of July 2024, the author was quoted a sales price of $34,000 including a PC, which was found to be well below every other suitable immunofluorescent scope being marketed. However, while this is a reasonable cost, it was the superior performance, software ease of use, and outstanding support from top management who freely took their time to help with new application that very much impressed this author.

3. Results

Testing the Etaluma LS820 Immunofluorescence Microscope

For Gel Immunodiffusion Assays

The Etaluma LS820 Immunofluorescence Microscope was tested using the Ouchterlony double immunodiffusion test first described by Orjan Ouchterlony in 1948 [3]. The test is run in clear agar in which wells are cut to accommodate various antigens or antibodies, and if there is reactivity, precipitin bands form similar to that shown here on a glass slide and also using a standard petri dish:

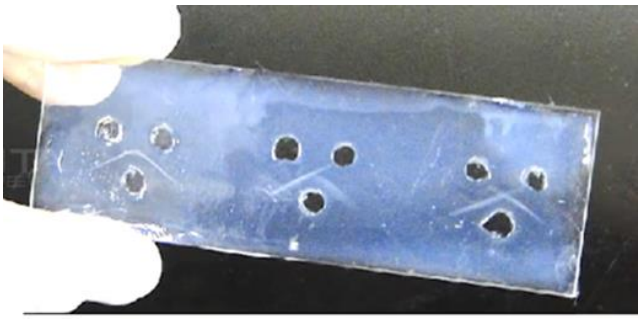
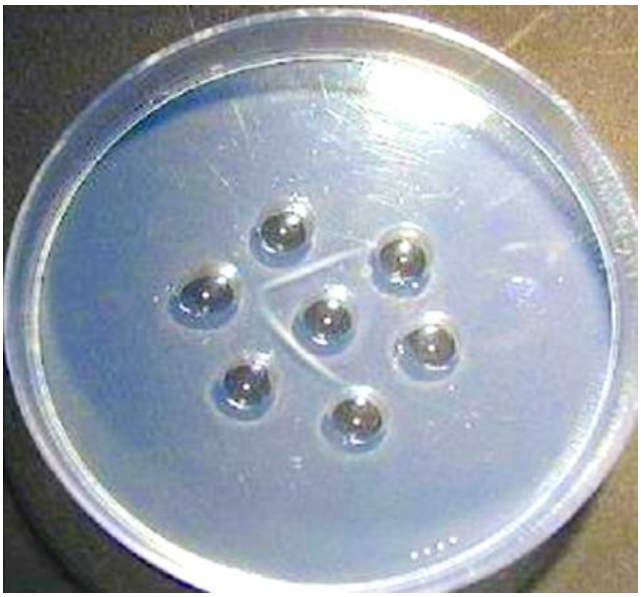
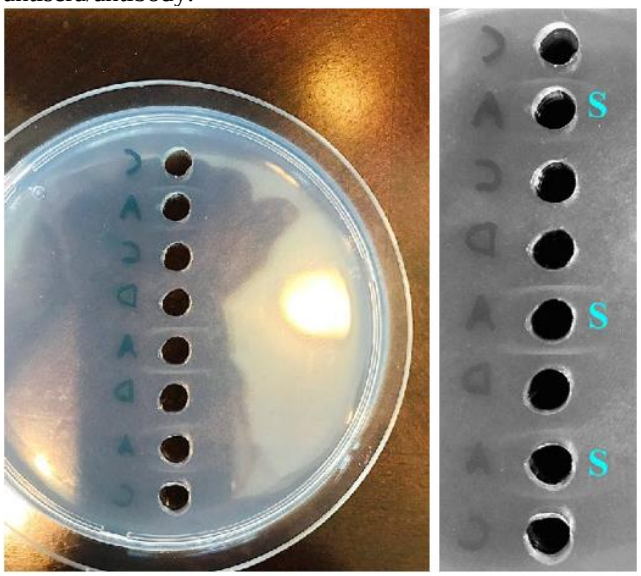


Figure 1



Figures 2

The test was modified by the author to utilize wells in a column formation so as to yield parallel precipitin bands for ease of reading the results with a scanner or microscope fitted with appropriate software; the wells marked "S" constitute the Saliva sample, all other wells contain antisera/antibody.



Figures 3 and 4, showing images in petri dish before and after cropping.

The use of petri dishes as opposed to the traditional glass slides yields ease of handling and stacking in an incubator.

The purpose of the inquiry using the Etaluma scope was to determine if the Ouchterlony Test could be converted to a quantitative test in order to evaluate saliva growth factors before and after a therapeutic trial.

Although both the "precipitin ring test" and the "radial immunodiffusion assay" give more quantitative results as to the amount of antibody present in serum or saliva, the requirement of massive amounts of antigen and/or antibody needed to perform the tests makes the procedures impractical for general saliva growth factor testing. Both antigen and antisera reagents are expensive and must be used judiciously in order reach an affordable assay for the individual practitioner, patient, or small lab.

The Etaluma immunofluorescence microscope "Detects blue, green and red fluorophores, including common probes such as Hoechst, DAPI, **FITC**, Fluo-4, GFP, Texas Red & mCherry GFP...Multi-OS software allows set-up and control across any locations, including microplates, microfluidic chips, **slides, dishes**, flasks, and deck-top chambers and custom arrays" and so, was and is very suitable for assaying many different types of fluorescent-labeled antibodies.

Saliva was collected for testing from family members using the "Passive Drool Method", well documented elsewhere [4]. The Ouchterlony Test was performed using the standard procedure well documented many times in the literature [5].

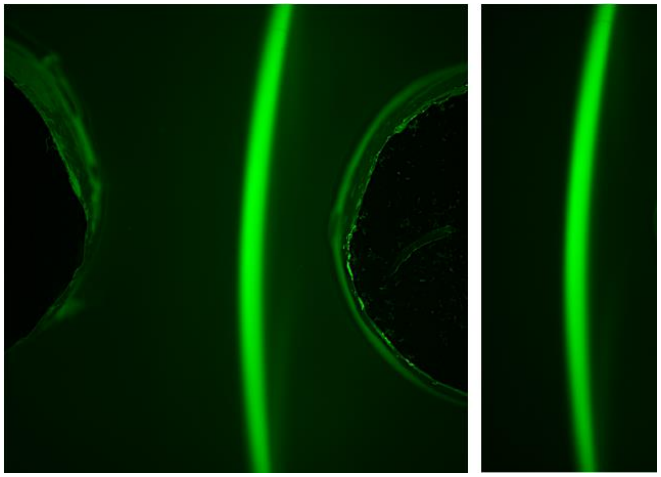
In this study, the author was privileged to have the collaboration of Chris Shumate, CEO of Etaluma Inc. The Ouchterlony tests were run using a variety of human saliva antigens, such as albumin, IgG, and IgA, against various fluorescent labeled antibody or antisera, typically

conjugated with FITC (fluorescein isothiocyanate) in order to test proof of concept.

The author conducted the tests using standard gel in petri dishes as noted above. Saliva was placed in various wells, with various antisera or antibody in alternating wells Maximum antigen-antibody precipitation bands typically form within 48 hours of incubation at 37°C. These remain fixed and stable for up to a week at room temperature. The plates were covered to avoid any photo bleaching from light, and shipped express to Etaluma for further work-up by Chris Shumate using the Etaluma Model LS820. Some bands that were faint or largely invisible to the naked eye could be visualized and quantified by use of the microscope detecting small quantities of fluorescence.

The bands could then be analyzed for fluorescent intensity that was related to the amount of precipitation achieved via the included software.

Example of fluorescent precipitin bands as observed:



Figures 5 and 6 showing band observed between two wells and cropped.

The various bands were visualized manually and quantification took place via the associated LumaviewPro multi-operating system software as detailed in the specs:

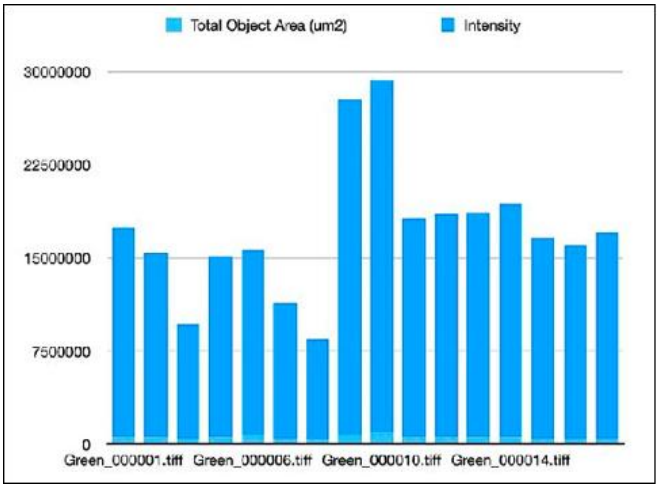
The system automatically reads and quantifies each precipitin band and gives an intensity number. In order to convert the intensities to actual concentrations, or relative concentration of antigen, the investigator must make use of a standard curve in which known quantity of antigen is loaded into a series of wells with antibody in alternating wells. This was not done in this review because it may not always be needed. Actual concentration of a saliva growth factor in saliva will vary from patient to patient. **In stem cell therapeutics, the inquiry is not what the individual growth factor concentration is in this or that patient, but rather, did the concentration change in response to a therapeutic. This is the important information sorely lacking in the advancing utilization of stem cell therapy.**

LS820 Microscope Specifications	
LED Excitation:	405 nm, 488 nm, and 589 nm
Fluorescence Filters:	Blue: Excitation 370-410 nm, Emission 429-462 nm Green: Excitation 473-491 nm, Emission 502-561 nm Red: Excitation 580-598 nm, Emission 612-680 nm
Transmitted Modes:	Bright Field, Phase Contrast
Objectives:	1.25x, 2.5x, 4x, 10x, 20x, 40x, 60x, and 100x(oil)
Camera:	High Sensitivity Monochrome CMOS BSI Sensor; 5 MP 12-Bit
Image Formats:	JPG, BMP, TIF, or PNG
Image Size:	Adjustable up to 2100 x 2100 pixels
Video Rates:	25 FPS (exposure limited)
Motorized Z:	14mm travel, 100 nm step, image-based autofocus, Z-stacks
Control Software:	LumaviewPro, multi operating system application
Computer Requirements:	Windows 10, 11; Core i7, 512TB SSD, 8GB RAM MacOS with M1; 512TB SSD, 8GB RAM
Linux Debian Distributions:	512TB SSD, 8GB RAM
Power Requirements:	80-264 V, 50-60 Hz 10W typical, 40W Max
Integration:	Python source under the MIT Open Source License
Dimensions:	24 cm x 22.6 cm x 27.8 cm
Weight:	5 Kg/11 lbs
Operating Conditions:	0°C - 42°C, 5% - 95% RH non-condensing

The following **Figure 7** data and generated bar graph represents the **typical output for the system**. Note that Area is also measured and this is extremely important because the precipitin bands can vary in width, length, and intensity:

Results

Precipitin Band Number	Total Object Area (um2)	Intensity
Green_000001.tiff	547725	16911596.0
Green_000003.tiff	603868	14772044
Green_000004.tiff	497563	9234522.7
Green_000005.tiff	585216	14577080.8
Green_000006.tiff	750764	14870736.6
Green_000007.tiff	389784	11011999.4
Green_000008.tiff	357694	8052504.3
Green_000009.tiff	839126	26983512.2
Green_000010.tiff	887208	28450633.6
Green_000011.tiff	541435	17603585.1
Green_000012.tiff	578794	18027193.7
Green_000013.tiff	553495	18123819.2
Green_000014.tiff	515528	18891452.4
Green_000015.tiff	438982	16184955.5
Green_000016.tiff	429520	15618224.4
Green_000017.tiff	421324	16722163.9



The following **Figure 8** demonstrates typical raw date for author's plates, which data was converted in one click to a Bar Graph using Mac Numbers software:

Typical Ouchterlony Raw Data Output

Band Number	Area (um2)	Intensity
1	1564088	70228087
2	597874	19008078
3	689369	22674999
4	617080	19401293
5	314323	16433752
6	440593	19306210
7	552928	18130571
8	525624	15432338

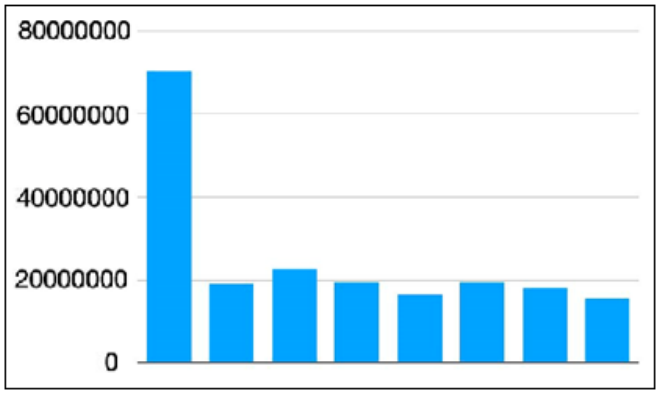


Figure 8: Typical Ouchterlony Raw Data Output

4. Discussion

The purpose of this inquiry was to determine if the Etaluma Model LS820 could be adapted for the testing of saliva growth factors and cytokines via the Ouchterlony double immunodiffusion technique using fluorescent-labeled antibody. We found the combination of hardware/software to provide not only an elegant solution to the quantification of antigen in saliva samples; but most importantly, an economical contribution to the field of saliva testing.

And it should also be emphasized again that absolute concentration of this or that growth factor in a patient's saliva is not so important as whether that amount changes up or down in response to a therapeutic modality.

For example, Neil H. Riordan's team have developed a nutraceutical that doubles circulating stem cells for patients *in vivo* within a 14 day period after supplementation [6]. It would be informative to observe if an increase in growth factors is associated with an increase in circulating stem cells, and if such growth factors, tested in patient's saliva in a serial fashion, preceded the stem cell release. If this were shown, then one could postulate that the increase in various circulating growth factors play a predominant role in stimulating the increase in endogenous stem cells. These and many other questions relating to stem cell therapeutics, red light therapy, Low Level Laser Therapy (LLLT), could have some evidentiary basis for effectiveness.

For another example, are increased growth factors recorded in saliva mini-liposuction alone the same levels as mini-liposuction along with the processing of the SVF (stromal vascular fraction) and re-injection of patient stem cells? These and many similar questions relating to stem cell therapeutics need to be answered as the field attempts to advance and prove effectiveness of therapies.

In the very early days of the development of the Ouchterlony double immunodiffusion test, and even through the 1970's, quantification was almost impossible to achieve even though some brave researchers attempted it [7,8]. The primary use of fluorescent microscopes in the 1970's was the qualitative detection of immune complexes for example associated with glomerular nephritis [9]. It has only been with the advent of analytic software that the quantitative measurement of antigen in Ouchterlony fluorescent precipitin bands can be achieved, thereby breathing new life and utility into this classic immunological test.

5. Conclusion

The Ouchterlony double immunodiffusion test coupled with the very economical yet highly sensitive Etaluma Model LS820 immunofluorescence microscope and software offers a pathway to saliva growth factor testing. Such testing is for purposes of evaluating stem cell and other therapeutic modalities in single-patient clinical trials. In such cases, serial samples of saliva, a non-invasive procedure, can be obtained and compared. Because the purpose of such testing is for therapeutic effectiveness, rather than for purposes of diagnosis, only relative concentrations of changes in antigen level are needed.

However, quantitative results can be achieved by using concentration curves developed by serial dilution of known quantity of antigen, and comparing intensities achieved with the patient saliva sample.

The primary problem with the Ouchterlony test has always been high variability of results due to operator variability. The Etaluma Model LS820 immunofluorescence microscope solves this issue by converting fluorescent precipitin bands to quantitative data, thereby offering reliable objective results. And the high sensitivity of the instrumentation allows very low quantities of protein to be recognized as well.

This report is important for suggesting an affordable saliva testing procedure for growth factors and cytokines that could be effectively utilized by physicians and other health care providers in an office setting. Naturopathic physicians, other alternative medicine practitioners, and small labs would also benefit from the techniques and instrumentation summarized here.

Acknowledgement

This work could not have been completed but for the generous collaboration offered by Chris Shumate, CEO, of Etaluma Inc. who gave his time and expertise freely to further the implementation of the experimental design and to demonstrate proof of concept.

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Author Profile

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Table Comparing Major Factors/Cytokines
(Copyright Walter P. Drake June 27, 2023)

Growth Factors/Cytokines	Adipose Tissue	SVF Cell Free Fraction	PRP	Amniotic Fluid
CTGF Connective Tissue Growth Factor	✓		✓	
ECGF Endothelial Cell Growth Factor			✓	
bFGF Basic Fibroblast Growth Factor	✓	✓	✓	✓
TGF β1 Transforming Growth Factor Beta 1	✓	✓	✓	✓
VEGF Vascular Endothelial Growth Factor	✓	✓	✓	✓
IGF 1&2 Insulin-like Growth Factors	✓	✓	✓	✓
PD-EGF Platelet Derived Epidermal Growth Factor			✓	
PDGF-α and β Platelet Derived Growth Factor	✓	✓	✓	✓
FGF-2 Fibroblast Growth Factor 2	✓		✓	✓
IL-6 Interleukin 6	✓	✓	✓	✓
IL-8 Interleukin 8	✓	✓	✓	✓
IL-1β Interleukin 1β	✓	✓	✓	
IL-10 Interleukin 10	✓	✓	✓	✓
IL-12p70 Interleukin 12p70			✓	
TNF-α Tumor Necrosis Factor Alpha	✓	✓	✓	✓
NGF Nerve Growth Factor	✓	✓	✓	
HGF Hepatocyte Growth Factor	✓	✓	✓	✓
EGF Epidermal Growth Factor	✓	✓	✓	✓
GCSF Granulocyte-Colony Stimulating Factor				✓
M-CSF Macrophage Colony Stimulating Factor				✓
FGF4,7,19,21 Fibroblast Growth Factors 4,7,19,21				✓
SCF Stem Cell Factor				✓
Fibrinogen			✓	
PF5 Platelet Factor 5			✓	
P-selectin			✓	
α-granules, dense granules, and lysosomes			✓	
			Over 300 proteins identified including 125 proteins related to wound healing; 4 for collagen biosynthesis;2 proteins for glycosami- noglycan biosynthesis process; 13 proteins for glycosaminoglycan binding. (27)	Over 300 cytokines reported in main categories of host defense, proliferation/ differentiation, cell adhesion/cell-cell interactions, angiogenesis (28)