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TITLE:

Quantification of Hypopigmentation Activity *In Vitro*

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SUMMARY:

We describe three experimental methods for evaluating the hypopigmentation activity of chemicals *in vitro*: quantification of 1) cellular tyrosinase activity and 2) melanin content, and 3) measurement of melanin by cellular melanin staining and image analysis.

ABSTRACT:

This study presents laboratory methods for the quantification of hypopigmentation activity *in vitro*. Melanin, the major pigment in melanocytes, is synthesized in response to multiple cellular and environmental factors. Melanin protects skin cells from ultraviolet damage, but also has biophysical and biochemical functions. Excessive production or accumulation of melanin in melanocytes can cause dermatological problems, such as freckles, dark spots, melasma, and moles. Therefore, the control of melanogenesis with hypopigmentation agents is important in individuals with clinical or cosmetic needs. Melanin is primarily synthesized in the melanosomes of melanocytes in a complex biochemical process called melanogenesis, which is influenced by extrinsic and intrinsic factors, such as hormones, inflammation, age, and ultraviolet light exposure. We describe three methods to determine the hypopigmentation activity of chemicals or natural substances in melanocytes: measurement of the 1) cellular tyrosinase activity and 2) melanin content, and 3) staining and quantifying cellular melanin with image analysis.

In melanogenesis, tyrosinase catalyzes the rate-limiting step that converts L-tyrosine into 3,4-dihydroxyphenylalanine (L-DOPA) and then into dopaquinone. Therefore, the inhibition of tyrosinase is a primary hypopigmentation mechanism. In cultured melanocytes, tyrosinase activity can be quantified by adding L-DOPA as a substrate and measuring dopaquinone production by spectrophotometry. Melanogenesis can also be measured by quantifying the melanin content. The melanin-containing cellular fraction is extracted with NaOH and melanin is quantified spectrophotometrically. Finally, the melanin content can be quantified by image analysis following Fontana–Masson staining of melanin. Although the results of these *in vitro* assays may not always be reproduced in human skin, these methods are widely used in melanogenesis research, especially as the initial step to identify potential hypopigmentation activity. These methods can also be used to assess melanocyte activity, growth, and differentiation. Consistent results with the three different methods ensure the validity of the effects.

INTRODUCTION:

Melanin plays a critical role in the physiology, pathology, and toxicology of several organs including the skin, eyes, and brain[1]. Major functions of melanin are photo-screening and biochemical effects. Melanin absorbs near-infrared and visible light as well as ultraviolet (UV) radiation, with increased absorption rates at shorter wavelengths of light; thus, melanin protects tissues from damage caused by visible light or UV radiation[2]. Melanin is an antioxidant and has an affinity for metals and other toxic chemicals; therefore, it can protect tissues from oxidative and chemical stress[3]. However, the excessive production of melanin causes dermatological issues.

The quantity and quality of melanin in the skin and iris are the most important determinants of the color of the iris and skin. Individuals may have different skin color preferences; some favor tanned skin, while others favor lighter skin colors. Depending on these consumer profiles, hypopigmentation cosmetics have been developed to satisfy individual markets for favored skin colors[4]. Accordingly, studies of hypopigmentation and anti-melanogenic activity are important both scientifically and practically.

Melanogenesis is the complex process of melanin biosynthesis through a series of enzymatic and spontaneous chemical reactions in melanocytes. One melanocyte is surrounded by approximately 36 keratinocytes and melanocytes are the melanin synthesis factories that distribute their product to neighboring keratinocytes. In skin, the melanin produced and stored in the melanosomal compartment of melanocytes is transported to neighboring keratinocytes in the epidermis *via* dendrites.

L-Tyrosine serves as the initial substrate for melanogenesis and the enzyme tyrosinase catalyzes two consecutive reactions that convert L-tyrosine into 3,4-dihydroxyphenylalanine (DOPA) and then into dopaquinone. These reactions are the rate-limiting step in melanogenesis[5,6]. Accordingly, hypopigmentation activity can first be measured by assessing cellular tyrosinase activity directly. To do so, melanocyte extracts containing tyrosinase are incubated with DOPA and the dopaquinone produced in the samples can be measured by spectrophotometry at 475

nm. The values are normalized by the protein concentrations of the samples, and substances with hypopigmentation activity result in less dopaquinone formation compared with controls.

Secondly, hypopigmentation activity can be quantified by measuring melanin in cultured melanocytes directly. After treating cells with the test material, melanin is extracted under alkaline conditions and the melanin content is quantified by spectrophotometry at 400 nm. A hypopigmentation agent will result in a lower melanin content than the controls[7].

Finally, the hypopigmentation activity can be quantified by Fontana–Masson melanin staining and subsequent image analysis. In Fontana–Masson staining, melanin granules reduce ammonia-silver nitrate to a visible black metallic state and the black areas of cells in microscopic images represent the amount of melanin.

A hypopigmentation agent usually gives consistent and comparable results with these three methods, which confirms that the activity of the substance is valid. Alternatively, it may be useful to measure the expression of key genes and proteins in melanogenesis in response to a test substance to examine hypopigmentation activity. In addition to tyrosinase, tyrosinase-related proteins (TRP-1) and dopachrome tautomerase (TRP-2) are critical enzymes in melanogenesis[8]. The transcription factor microphthalmia-associated transcription factor (MITF) is a master regulator in melanogenesis and quantification of its gene/protein expression levels or a promoter activity assay can also be used to assess hypopigmentation activity.

PROTOCOL:

1. Preparation of Medium, Compounds, and Reagents

1.1. Prepare B16F10 growth complete medium. Supplement Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PEST).

1.1.1. To make 500 mL of complete medium, mix 445 mL of Dulbecco's modified Eagle's medium (DMEM) with 50 mL of FBS and 5 mL of PEST in a 1 L sterilized glass bottle. After mixing gently, filter the medium through a 0.2-µm bottle-top filter to a new sterilized glass bottle and then store at 4 °C.

CAUTION: All cell culture techniques should be undertaken in microbiological safety cabinet using aseptic technique to ensure sterility.

1.2. Prepare lysis buffer: 20 mM Tris (hydroxymethyl) aminomethane containing 0.1% Triton X-100 (v/v) buffer at pH 7.5 (pH adjusted with HCl). This buffer can be used for 3 months when stored at room temperature (RT). Add protease inhibitors to the lysis buffer right before use.

1.3. Prepare tyrosinase inhibitor assay buffer. Prepare 1 M NaH_2PO_4 (monobasic) and 1 M Na_2HPO_4 (dibasic) stock solutions. Mix 46.3 mL of Na_2HPO_4 and 53.7 mL of NaH_2PO_4 to prepare a final volume of 0.1 M sodium phosphate buffer (pH 6.8).

1.3.1. Dilute the resulting mixture to 1 L (final volume) with H_2O. Adjust the pH of the final solution to 6.8. This buffer can be used for 1 month when stored at 4 °C.

1.4. Prepare tyrosinase substrate solution: 0.1% 3,4-dihydroxy-L-phenylalanine (DOPA, w/v) dissolved in tyrosinase inhibitor assay buffer (refer to Step 1.3).

CAUTION: Keep on ice while in use.

1.5. Optionally, prepare 100 U/mL mushroom tyrosinase. Dissolve the lyophilized mushroom tyrosinase in tyrosinase inhibitor assay buffer. This solution can be used for 2 months when stored at –20 °C. The mushroom tyrosinase activity as well as cellular tyrosinase activity are calculated in the presence of cell materials.

CAUTION: The solution is aliquoted before freezing thus repeated freezing-and-thawing can be avoided. Keep the solution on ice while in use.

1.6. Prepare 1 N NaOH solution. Weigh 4 g of NaOH in a volumetric flask and dissolve it in distilled water to make 100 mL.

1.7. Prepare fixation solution (10% formalin). Dilute 27 mL of 37% formaldehyde (10% formalin) with 73 mL of distilled water. Prepare fresh daily.

1.8. Prepare ammoniacal silver stock solution. Prepare 5 mL of 10% silver nitrate solution in a volumetric flask. Add ammonium hydroxide solution dropwise, until the precipitate is completely dissolved. Add 200 µL of silver nitrate solution (10%). The solution will become slightly cloudy.

CAUTION: Avoid contact and inhalation. Use a fume hood.

1.9. Prepare ammoniacal silver working solution. Dilute 2.5 mL of ammoniacal silver stock solution with 7.5 mL of distilled water. Use once and then discard.

CAUTION: Avoid contact and inhalation. Use a fume hood.

1.10. Prepare 0.1% gold chloride. Prepare 1 mL of 10% gold chloride and add 99 mL of distilled water in a volumetric flask. Prepare fresh daily.

1.11. Prepare phosphate buffer saline (PBS). Add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 mL of distilled water. Adjust the pH to 7.4 with HCl. Dilute the resulting mixture to 1 L (final volume) with distilled water.

1.12. Prepare 5% (w/v) sodium thiosulfate solution dissolved in distilled water.

2. Cell Culture and Treatment

2.1. Use commercially available B16F10 cells. Maintain the B16F10 cells in complete medium. Keep the cell culture flask in a humidified, 5% CO_2 atmosphere incubator at 37 °C.

2.2. Prepare test compounds, inhibitor positive control, and negative control samples.

2.2.1. Dissolve the test compounds in the appropriate solvents. Dissolve water soluble compounds in double-distilled water. Dissolve water insoluble compounds in appropriate organic solvent, *e.g.*, DMSO.

2.2.2. Here, use arbutin dissolved in double-distilled water (125 µg/mL) as a positive control to assess the hypopigmentation activity compared with test compounds or a negative control. As a negative control (vehicle-treated control), use the solutions or buffers used in the test sample, *e.g.*, double-distilled water, DMSO, ethanol.

2.3. Cell seeding and treatment

2.3.1. Seed B16F10 cells at 5×10^4 cells/well in 24-well culture plates using complete medium and incubate in a CO_2 incubator for 24 h. After incubation, aspirate the complete medium.

2.3.2. Incubate cells with DMEM medium (without FBS and 1% penicillin/streptomycin) containing either test compounds, an inhibitor control, or a negative control.

2.3.3. Add test compounds at the desired concentration for 72 h. Check cells every 24 hours under the microscope. After this step, go to Steps 3-5 for further experiments.

CAUTION: The mixture of the test substance and DMEM medium should be filtered through a 0.2 µm filter.

3. Measuring Cellular Tyrosinase Activity

3.1. Using the method described in Step 2, remove media and rinse cells twice with 300 µL of cold PBS. Then aspirate the PBS on a clean bench.

3.2. Place the cell culture plate on ice. Add 300 µL of lysis buffer and incubate for 5 min. Then collect the cell lysate using a cell scraper and transfer the cell lysate to a 1.5-mL microcentrifuge tube.

3.3. Homogenize the cell lysate with a cell homogenizer at 14,500 rpm for 2 s, 3 times on ice to obtain intracellular tyrosinase. Use the optimum condition specific for the homogenizer.

3.4. Centrifuge for 10 min at 12,000 × *g* at 4 °C and transfer the cell supernatant to a 1.5-mL microcentrifuge tube. Keep on ice while in use.

3.5. Transfer 70 µL of the supernatant to a 96-well clear polystyrene microplate.

3.6. Add 140 µL of a solution containing tyrosinase substrate (DOPA) to a 96-well clear polystyrene microplate, shake gently, and incubate for 2 h at 37 °C.

3.7. Optional, add 70 µL of 100 U/mL mushroom tyrosinase solution to each well and incubate for 2 h at 37 °C.

3.8. Measure the tyrosinase activity at a wavelength of 475 nm using microplate reader.

3.9. Normalize the tyrosinase activity to the protein concentration of each sample. Measure the protein concentration of each sample by a Bradford assay.

4. Measuring Melanin Content

4.1. Using the method described in the Step 2, remove media and rinse cells twice with 300 µL of cold PBS. Then aspirate the PBS on a clean bench.

4.2. Place the cell culture plate on ice. Add 300 µL of lysis buffer and incubate for 5 min. Then collect the cell lysate using a cell scraper and transfer the cell lysate to a 1.5-mL microcentrifuge tube.

4.3. Centrifuge for 10 min at 12,000 × *g* at 4 °C.

4.4. Transfer the supernatant to a new tube and measure total protein concentration for normalization. Measure the protein concentration of each sample by a Bradford assay.

4.5. Add 300 µL of 1 N NaOH to each pellet and incubate at 60 °C for 1 h.

4.6. Centrifuge the dissolved solution for 5 min at 12,000 × *g* at 4 °C.

4.7. Transfer 200 µL of the supernatant to a 96-well microplate.

4.8. Measure the melanin contents at a wavelength of 400 nm.

4.9. Normalize the melanin contents to the protein concentration.

5. Fontana–Masson staining

5.1. Fontana–Masson staining

5.1.1. Wash cells twice with 300 µL of cold PBS.

5.1.2. Add 200 µL of 10% formalin to each well and incubate for 1 h at 4 °C.

5.1.3. Rinse with distilled water.

5.1.4. Add 200 µL of pre-warmed ammoniacal silver working solution and incubate for 1 h at 37 °C or until the cells become yellow/brown in color.

CAUTION: Place freshly mixed ammoniacal silver working solution in a 58–60°C water bath and allow adequate time for the temperature to equilibrate.

5.1.5. Remove ammoniacal silver working solution and rinse for 2 min with distilled water.

5.1.6. Remove distilled water. Add 200 µL of 0.1% gold chloride and incubate for 2 min at RT.

5.1.7. Remove 0.1% gold chloride solution and rinse for 2 min with distilled water.

5.1.8. Remove distilled water. Add 200 µL of sodium thiosulfate solution (5%, w/v) and incubate for 2 min.

5.1.9. Remove sodium thiosulfate solution and rinse with distilled water.

5.1.10. Remove distilled water and add 200 µL of Nuclear Fast Red for 5 min.

5.1.11. Remove Nuclear Fast Red and rinse for 2 min with tap water.

5.1.12. Remove tap water and observe the stained cells under a microscope.

5.2. Fontana–Masson staining (Threshold Analysis Using ImageJ)

Note: An example of the image analysis procedure using ImageJ software is shown in the Supplemental Materials.

5.2.1. Open the program ImageJ.

5.2.2. Select **File |Open |Microscope Image**.

5.2.3. Select **Image | Adjust | Color Threshold.**

5.2.4. To measure the area stained with Fontana–Masson, set the brightness parameter bar to zero and adjust the brightness bar to find the point at which all stained cells (black or dark brown) are included.

5.2.5. Select **Analyze | Analyze Particles**. Check the summarize box in the analyze particles window and press **OK**. Obtain the summary result and paste into a spreadsheet.

Note: The description of the resulting box parameters are as follows:
Slice: Name of each image
Count: Number of stained cells
Total Area: Total area of the counted cells
Average Size: Total Area/Count
% Area: Stained Area/Total Area × 100%; total area is calculated automatically.

5.2.6. Calculate the relative area stained with melanin using the value of Total Area.

Relative melanin stained area (%) = Value of total cellular area of test sample/average total area of control × 100, *i.e.,*

REPRESENTATIVE RESULTS:

Representative results for the hypopigmentation activity of arbutin, an anti-melanogenic compound, in B16F10 melanocytes are shown below. **Figure 1A** shows that arbutin significantly suppressed the cellular tyrosinase activity compared with the vehicle-treated control. Similarly, the melanin content of cells stimulated with arbutin was significantly reduced compared with the controls (**Figure 1B**). Microscopic images of cells stained with Fontana–Masson stain are shown in **Figure 1C**. Arbutin treatment decreased the area of black pigment compared with the control.

FIGURE AND TABLE LEGENDS:

Figure 1: **Arbutin suppressed melanogenesis in melanocytes.** B16F10 cells were treated with arbutin (125 μg/mL) for 72 h. **A.** Cellular tyrosinase activity. **B**. Melanin content. **C**. Melanin was visualized with Fontana–Masson stain. Data are the means ± SEM (n = 4). Statistical comparisons were performed using Student's t-test (*p* values<0.05 were considered to be statistically significant).

DISCUSSION:

We presented protocols for evaluating the hypopigmentation activity of test compounds using cultured melanocytes. The representative results showed the hypopigmentation effect of arbutin, a tyrosinase inhibitor that inhibited tyrosinase activity and cellular melanin content. These methods are widely used in anti-melanogenic activity research. Using these assays, we have also successfully identified several bioactive compounds that have melanogenesis inhibitory effects on B16F10 cells in the past decade[9–14].

Melanin is produced by melanosomes in various pigment cells, including melanocytes in skin. In this study, we used B16F10 melanoma cells that produce melanin, which causes the culture medium to become dark brown or black[15]. Primary melanocytes might be biologically important tools for studying *in vivo* effects, but due to their limited proliferative capacity and the variability in phenotypes generated, cultured melanocytes are considered a reliable *in vitro* model[16]. Melanogenesis of this cell line is comparable with those of primary melanocytes. This method better represents the cellular environment than the mushroom tyrosinase activity

assay, in which the enzyme is directly incubated with the substrate and hypopigmentation test compound.

Before performing these experiments, it might be necessary to perform the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay or comparable assays, such as the cell violet assay, with the test compound(s). If a test substance affects cell proliferation or exhibits cytotoxicity, then melanocyte growth could be affected, and the results might not correctly represent the cellular tyrosinase activity. Chemicals or natural substances often have some cytotoxicity at high concentrations and this is particularly important for substances with mild activity. It is also not uncommon for substances and natural extracts to promote cell proliferation and differentiation. Consequently, the potential for additional bioactivity causing cell growth should be examined, although these effects on cell growth could be adjusted by normalizing the activity with the protein concentration.

Various mechanisms could result in hypopigmentation independent of tyrosinase activity. Melanogenesis is a complex biosynthetic pathway that involves multiple signal transduction pathways and transcription factor activities[17]. Therefore, the expression of key genes and proteins and melanogenic signal transduction pathways could be examined for an in-depth study of hypopigmentation. MITF is an important transcription factor in melanogenesis and α-melanocyte-stimulating hormone (α-MSH) can stimulate melanogenic signal transduction pathways[18,19]. Depending on their known biological characteristics, related signal transduction pathways could be studied further.

When measuring melanin content, the obtained values should also be normalized by the protein concentration, so that the results correctly represent the melanin content per cell or per culture area[20]. Additionally, phenol-red-free medium is recommended in the compound treatment step to avoid the negative effects of phenol red on absorbance.

For melanin staining, we used the Fontana–Masson stain, which detects argentaffin substances, such as melanin and argentaffin cells[21]. This technique is also used for paraffin or frozen section samples following the deparaffinization step. The reagents used in this experiment are harmful; contact and inhalation should be avoided, and the reagents should be used only in a fume hood whenever possible. After staining, ImageJ is used to calculate the stained area. With Fontana–Masson staining, it can be difficult to interpret faint staining in sparsely positive cells; however, this may not be an issue for melanin-rich melanocytes. The silver nitrate, ammoniacal silver, sodium thiosulfate, and ammonium hydroxide used in the experiments should be handled with care due to their toxicity.

These are widely used are methods in the field of melanogenesis and hypopigmentation because they are rapid, handy, cost-effective, and reliable. These methods may be useful for investigators who want to identify novel compounds or extracts with anti-melanogenic or pro-melanogenic activities. They could also be used to assess melanocyte cell growth or cellular activity. One limitation is the reproducibility of the results on human skin, which is a complex organ in which homeostasis is maintained in response to various environmental, chemical, and

biological factors. Therefore, hypopigmentation agents confirmed by these methods *in vitro* might not have significant effects on human skin *in vivo*.

In summary, these protocols are an important initial step in screening test compounds for their inhibitory effects on melanogenesis. We suggest that these methods can explain not only the inhibitory effects on melanogenesis but also the mechanism involved. After bioactive candidate compounds are identified, further testing may lead to studies of the biological mechanisms or commercial applications, followed by *in vivo* use.

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DISCLOSURES:

The authors have no disclosures to report.

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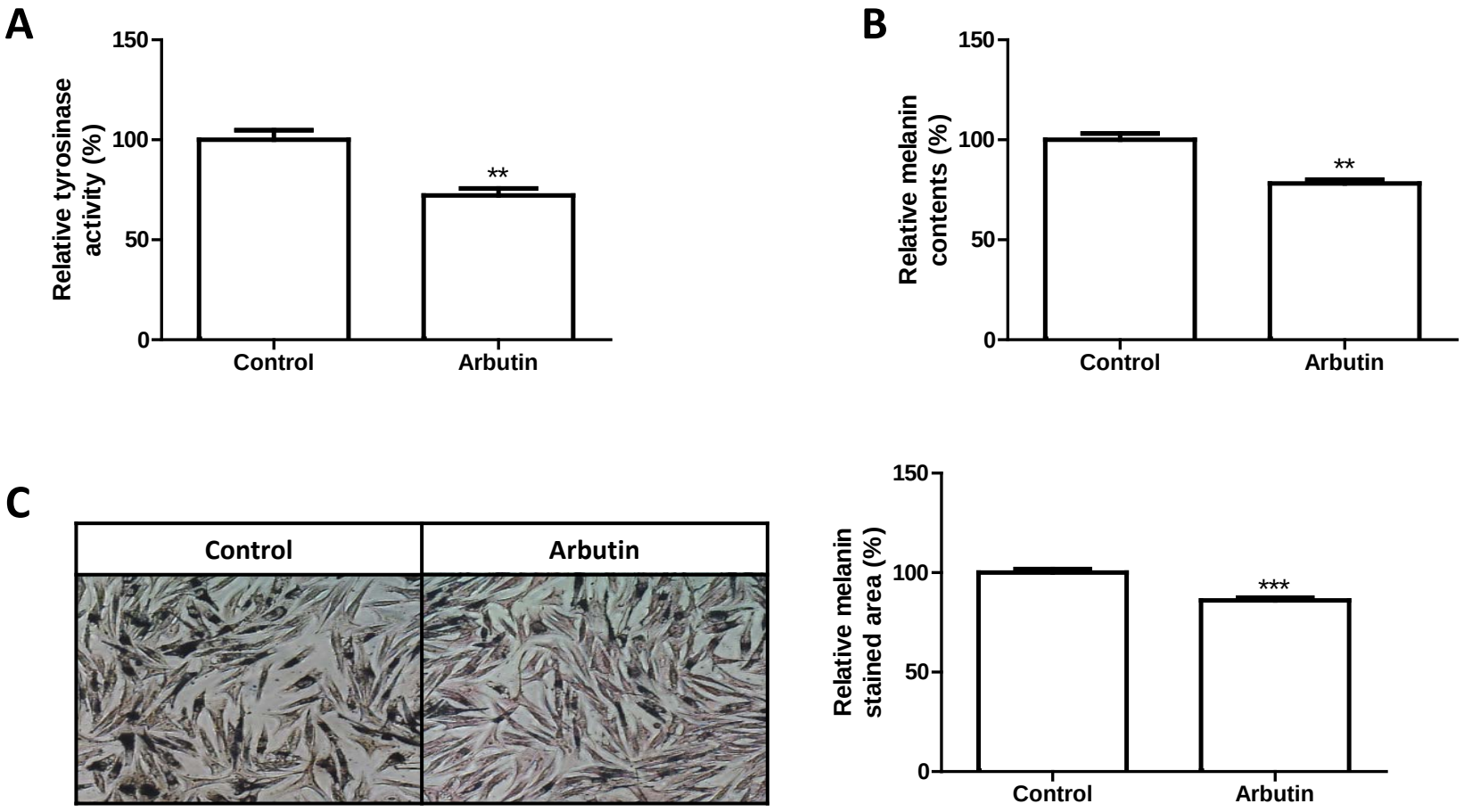
Figure

Figure 1



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3,4-Dihydroxy-l-phenylalanine	Sigma	D9628	
Ammonium hydroxide solution	Sigma	#320145	
Arbutin	Fluka	#10960	
DMEM	HyClone	SH30243.01	
FBS	HyClone	SH30084.03	
Formalin	Yakuri Pure Chemicals	#16223	37%
Gold chloride	American MasterTech	AHG0226	0.10%
HCl	Samchun chemical	H0255	
KCl	Bio basic Canada Inc.	PB0440	
KH_2PO_4	Sigma	#60218	
Na_2HPO4	J.T.Baker	#3817-01	
NaCl	Duksan pure chemicals	#81	
NaOH	Sigma	655104	
Nuclear fast red	Merck	100121	Nuclear fast red 0.1% in 5% aluminum sulfate.
Penicillin and streptomycin solution	HyClone	SV30010	
Silver nitrate	Duksan Pure Chemicals	#900	
Sodium phosphate dibasic (Na2HPO4)	J.T. Baker	#3817-01	
Sodium phosphate monobasic (Na2HPO4)	Sigma	S5011	
Sodium thiosulfate pentahydrate	Duksan Pure Chemicals	#2163	
Tris(hydroxymethyl)aminomethane	Bio Basic Canada	TB0196	
Triton X-100	Union Carbide	T8787	
Tyrosinase from mushroom	Sigma	T3824	25 KU

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Article Title:	Measuring hypopigmentation activity in cultured melanocytes
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Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We carefully reviewed the manuscript. Thanks.

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please revise lines: 62-69, 71-75,

Response: We revised the manuscript comply with editor's comments.

3. Please define the error bars in all of the figures: SD, SEM, etc.

Response: all data were expressed as the mean **±** SEM**.**

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Response: We revised 3.2 section in text. Eppendorf tube to microcentrifuge tube.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: We revised the manuscript in response to the reviewer's comment.

6. 2.2: What test compounds are used and at what concentrations? We need specific examples.

Response: We used arbutin (125 μg/mL) for inhibitor control. And we revised 2.2 section in text.

7. 2.3: How is the treatment done?

Response: We revised 2.3 section in text.

8. Please provide the composition of all solutions used.

Response: We added 1.11 and 1.12 section in text.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the summary author must add the usefulness of these protocol over other available protocols

Response: We added brief information in the text (line 366-369).

There are several

Major Concerns:

In the discussion section authors must compare the protocols with other protocols available before claiming that these protocols are more reliable, cost effective ,handy and rapid.

Precautions must be incorporated at the end of each protocol.

Response: The method described in the manuscript is very common and widely used method in this field, which is also cost effective, handy, and rapid. We added this information in the manuscript (line 366-369).

Minor Concerns:

In the manuscript there is repetition of some paragraphs (Line 27 to 29 and 78 to 81) that should be removed.

Response: We revised lines 80-81 in the text**.**

In the protocols section language should be more clear and understandable (Lines 119,123,127,129,146)

Response: We revised methods in the text (please see sentences in yellow highlights).

Legend of figure should be more informative

Response: We revised the figure legends.

Reviewer #2:

Manuscript Summary:

Three standard methods for evaluating the hypopigmentation activity of chemicals are clearly described in this manuscript. Biochemical procedures and image analysis, which are both researcher-friendly methods, are shown here. I believe this manuscript is useful for researchers.

Minor Concerns:

-Line 88-91 should be revised to correctly refer Cooksey et al (5). Because it suggests that an immediate metabolite of L-tyrosine produced by tyrosinase-catalyzed oxidation is dopa-quinone.

Response: According to Cooksey et al., they mentioned that there has been some controversy in the literature regarding the method of generation of DOPA. DOPA is formed directly by the hydroxylation of tyrosine, whereas DOPA is formed indirectly.

According to the indirect theory, dihydroxy derivatives involve formation by nucleophilic attack on dopaquinone, either by external nucleophiles (e.g. thiols such as cysteine) or by nucleophilic groups attached to the quinone as in the case of DOPA, where the side chain amino group acts as an intramolecular nucleophile. The nucleophilic property of the amino group is due to the lone pair electrons on the nitrogen, and attack on the ring by the amino group is followed by re-aromatization, involving hydrogen transfer to give the corresponding catechol as shown in Reaction 1. Evidence from pulse radiolysis experiments (5) indicates that 5,6-dihydroxyindolene (cyclodopa) reacts rapidly with dopaquinone to give rise to two products, DOPA and dopachrome (Reaction 2). Thus, according to this scheme, DOPA in essence arises by disproportionation of dopaquinone (DQ), i.e. 2 DQ3DOPA 1 dopachrome, and is not a direct product of tyrosine hydroxylation by tyrosinase. If Reaction 2 is correct, it should be possible

-Procedure written in line 163 does not give 0.1 % solution.

Response: We revised 1.10 section in text.

-Line 179 Reason why FBS and 1 % P/S is removed should be described.

Response: There are several ways to treat cell. In this protocol, we tried to exclude unexpected effect of treatment. The presence of undefined constituents in serum may enhance or suppress the effect of the tested drug or toxins. In addition, antibiotics can occur competition effects to test compound. So we routinely used serum free medium without antibiotics for cell treatment.

-Line 181 Method for measuring cellular tyrosinase activity written here will cause

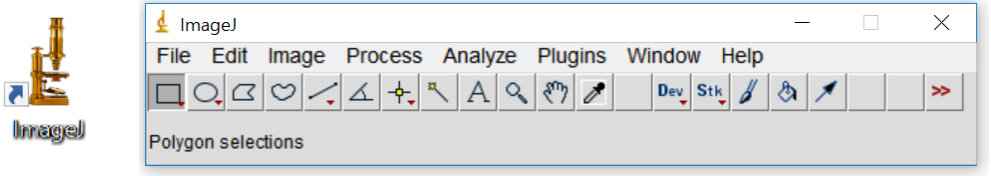
misunderstanding. The effect of tested materials on the down-regulation of tyrosinase expression cannot be excluded based on this method. Direct inhibition of tyrosinase enzyme should be clearly separated from it.

Response: We changed the title as in vitro assay.

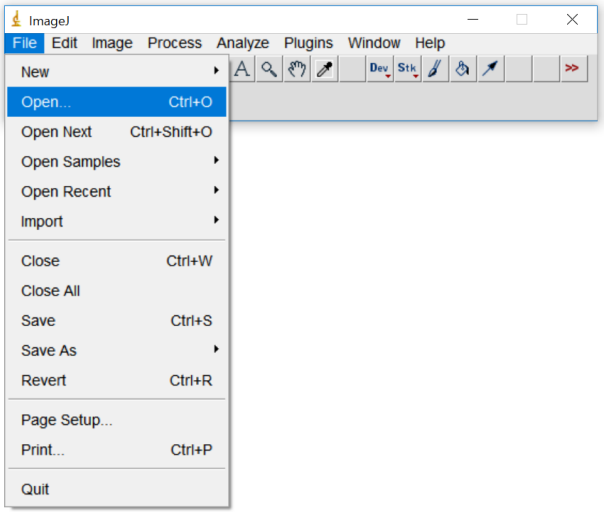
-Line 198 The optional procedure is performed to what? Addition of the enzyme will hide the contribution of intracellular tyrosinase.
Response: We agree with your opinion but B16F10 cells are unstable to produce melanin when cells are confluent.

Threshold analysis using Image J program

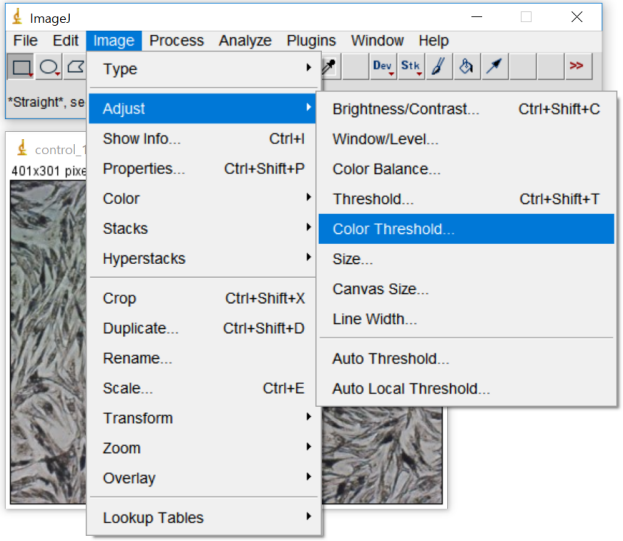
5.2.1.



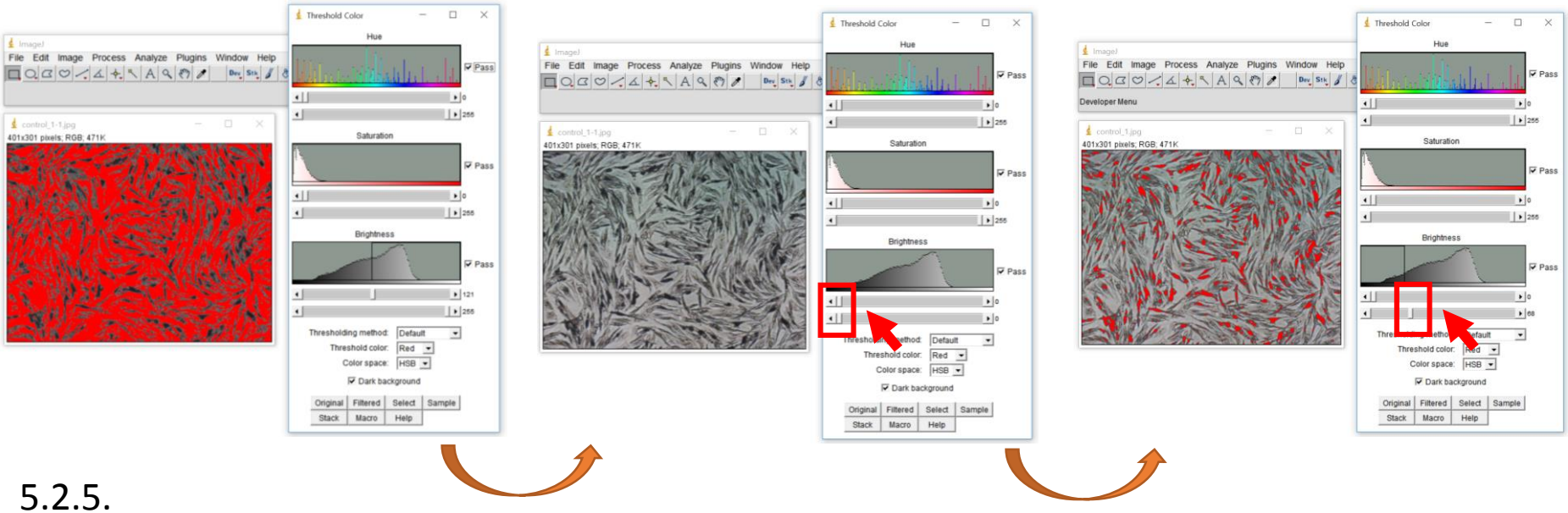
5.2.2.



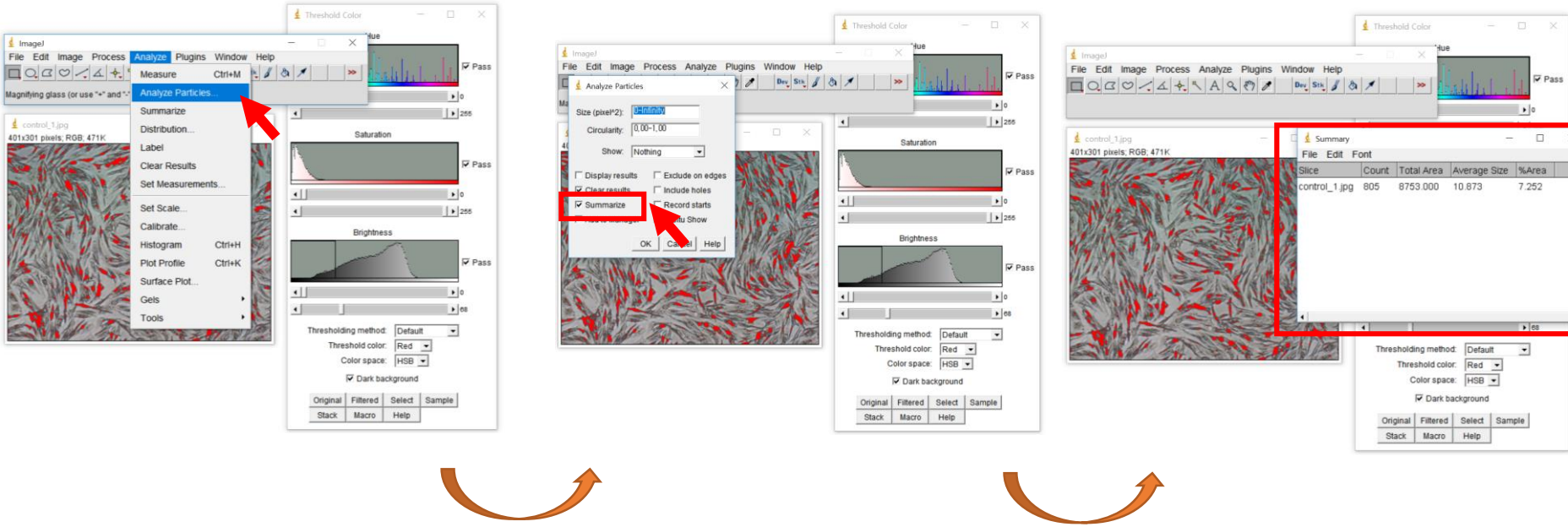
5.2.3.

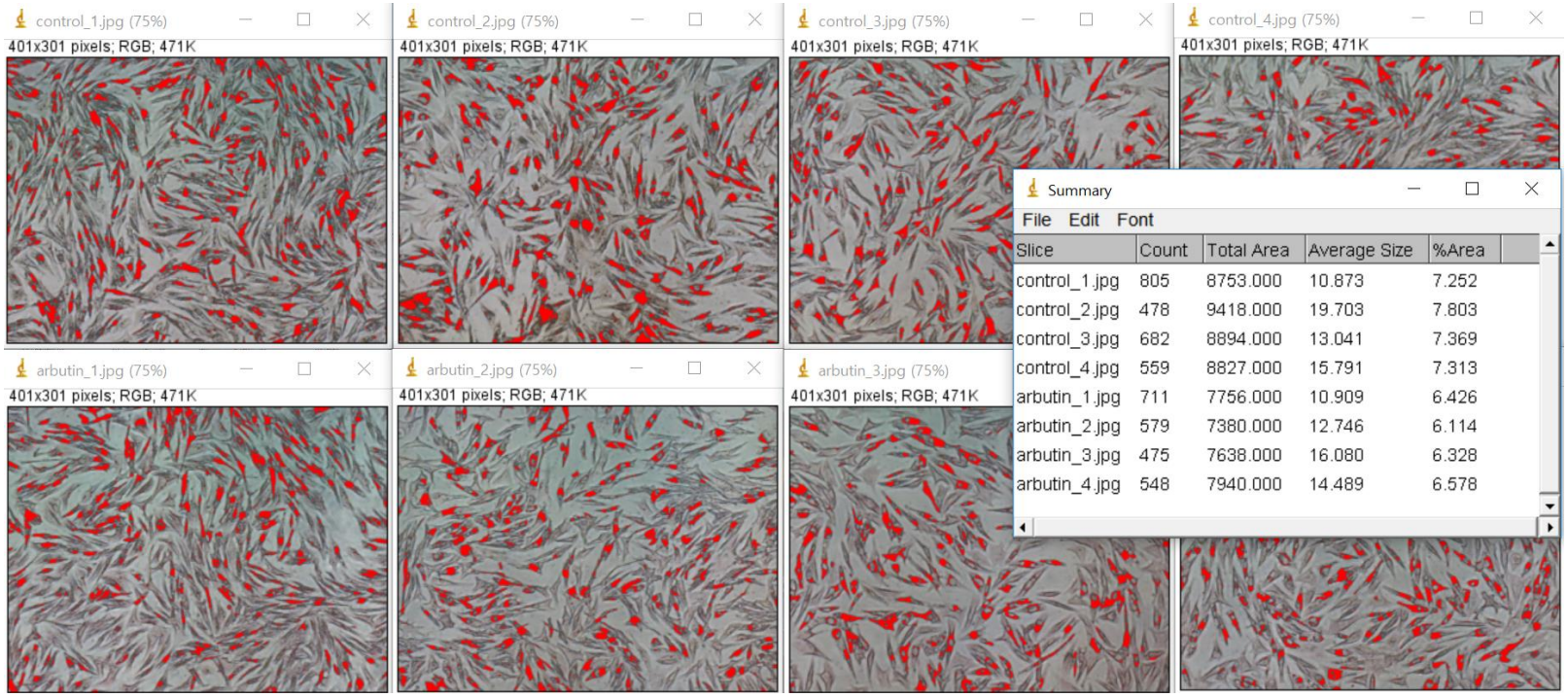


5.2.4.


ImageJ
File Edit Image Process Analyze Plugins Window Help
control_1-1.jpg
401x301 pixels; RGB; 471K
Threshold Color
Hue
Saturation
Brightness
Pass
Thresholding method: Default
Threshold color: Red
Color space: HSB
Dark background
Original Filtered Select Sample
Stack Macro Help
Developer Menu
control_1.jpg

5.2.5.


ImageJ
File Edit Image Process Analyze Plugins Window Help
Measure Ctrl+M
Analyze Particles...
Summarize
Distribution...
Label
Clear Results
Set Measurements...
Set Scale...
Calibrate...
Histogram Ctrl+H
Plot Profile Ctrl+K
Surface Plot...
Gels
Tools
Analyze Particles
Size (pixel^2): 0-Infinity
Circularity: 0.00-1.00
Show: Nothing
Display results
Exclude on edges
Include holes
Record starts
Summarize
OK Cancel Help
Summary
File Edit Font
Slice Count Total Area Average Size %Area
control_1.jpg 805 8753.000 10.873 7.252
Threshold Color
Hue
Saturation
Brightness
Pass
Thresholding method: Default
Threshold color: Red
Color space: HSB
Dark background
Original Filtered Select Sample
Stack Macro Help



Slice	Count	Total Area	Average Size	%Area
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control_2.jpg	478	9418.000	19.703	7.803
control_3.jpg	682	8894.000	13.041	7.369
control_4.jpg	559	8827.000	15.791	7.313
arbutin_1.jpg	711	7756.000	10.909	6.426
arbutin_2.jpg	579	7380.000	12.746	6.114
arbutin_3.jpg	475	7638.000	16.080	6.328
arbutin_4.jpg	548	7940.000	14.489	6.578