Journal of Visualized Experiments

Evaluation of the color difference in placentas with twin anemia–polycythemia sequence

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61312R2
Full Title:	Evaluation of the color difference in placentas with twin anemia–polycythemia sequence
Section/Category:	JoVE Medicine
Keywords:	monochorionic twin placenta, color difference, twin anemia polycythemia sequence
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Additional Information:	
Question	**Response**
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Leiden, The Netherlands

TITLE:

Evaluation of Color Difference in Placenta with Twin Anemia Polycythemia Sequence

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KEYWORDS:

monochorionic, twin anemia polycythemia sequence, TAPS, placenta, color difference, analysis

SUMMARY:

In monochorionic twins with twin anemia polycythemia sequence (TAPS), the donor twin and its corresponding placenta share are pale, while the recipient twin and its placental share have a plethoric aspect. Presented here is a protocol to quantify the color difference in maternal side of TAPS placenta after birth.

ABSTRACT:

Twin anemia polycythemia sequence (TAPS) occurs in 5% of monochorionic twins and is characterized by large inter-twin hemoglobin differences. The postnatal diagnostic criteria for TAPS are based on hematologic parameters and placental characteristics. Placental examination after birth shows that color of the maternal side between placental territories of the anemic and polycythemic twins is remarkably different. The color difference in TAPS placentas is higher compared to monochorionic placentas with acute peripartum feto-fetal transfusion; thus, this is used as an additional diagnostic criterion for TAPS. Software such as ImageJ enables the computer-based measurement of color intensity in TAPS placentas. However, a detailed

method for the calculation of color differences between anemic and polycythemic components of TAPS placentas has not yet been described. The protocol presented here provides a step-by-step method for analyzing color differences in the maternal side of TAPS placentas using ImageJ software.

INTRODUCTION:

TAPS is a chronic form of feto-fetal transfusion syndrome and characterized by a large inter-twin hemoglobin (Hb) difference without signs of oligo-polyhydramnios sequences[1]. The main pathogenesis of TAPS is related to the unique placental angioarchitecture, with only a few minuscule anastomoses allowing for chronic and low velocity transfusion from the donor (anemic twin) to recipient (polycythemic twin)[2-4]. Postnatal diagnosis of TAPS is based on hematological tests showing a large Hb difference and large reticulocyte count difference and/or placental injection with dye showing only minuscule anastomoses[5]. However, reticulocyte count is not always measured at birth, and placental injection is not routinely performed in most fetal medicine centers. An additional diagnostic criterion may be useful to help diagnose cases without reticulocyte count measurements and without placental dye injection. A striking color difference between the pale skin of a donor twin and the plethoric skin of the recipient twin is consistently present in TAPS twins at birth. A similar color difference of the maternal side between placental territories of the anemic and polycythemic twins is also detected upon gross examination.

It has been recently hypothesized that this placental color difference may be used as a simple and accurate method to confirm a TAPS diagnosis after birth[6]. The quantification of color difference in TAPS placentas is timesaving compared to placental injection. Additionally, quantification of color difference in TAPS placentas is more cost-effective compared to reticulocyte count. The materials used in this method are easily available in clinical settings Therefore, this method provides simple and accurate criteria for the diagnosis of TAPS. The protocol describes a detailed method for the quantification of color differences from the maternal side of monochorionic placentas with TAPS.

PROTOCOL:

This protocol was approved by the Leiden University Medical Center Ethics committee, and postnatal examination of human monochorionic twin placentas is a part of standard care at the Leiden University Medical Center. The acquisition of consent for postnatal examination of human monochorionic twin placentas is waived at the Leiden University Medical Center.

1. TAPS placenta collection and gross examination

1.1. Use all human monochorionic twin placentas with TAPS delivered at any gestational age. Keep the human monochorionic twin placenta as intact as possible. Deliver the monochorionic placenta as gently as possible, if manual removal is necessary.

NOTE: After birth, midwives or neonatologists should transfer TAPS placentas into a plastic box and then to the laboratory.

1.2. When the first infant is delivered, use one clamp to clamp the umbilical cord to mark the birth order as first. When the other infant is delivered, use two clamps to clamp the umbilical cord to mark the birth order as second.

1.3. Examine the placenta macroscopically, including placenta intactness, types of umbilical cord insertions, number of umbilical vessels, and gross examination of the dividing membrane to confirm the chorionicity. Collect the inter-twin membrane for pathological diagnosis of monochorionicity. The diameter of TAPS placentas is dependent on gestational age.

NOTE: Examination should be performed within 1 week after delivery to avoid the formation of adhesive blood clots on maternal side.

1.5. Keep the placenta refrigerated (1–4 °C) in a dry, plastic box until examination, if needed.

2. Preparation of the placental maternal side

2.1. Trim the amniotic membrane along the placental edge using scissors. Pay attention to the vessels on the membrane and if the velamentous cord insertion is present to prevent blood from flowing out of the placental mass.

NOTE: In velamentous cord insertion, vessels from the umbilical cord move to the surface of the amniotic membrane for some distance before attaching to the placental mass. If the “naked” vessels are cut, the blood from placental mass may flow out and make a mess.

2.2. Remove blood clots carefully to prevent damage to the placental lobes.

2.3. Organize the placental lobes on the maternal side to eliminate the gap between lobes. Placentas usually have around 15 lobes and can be identified on the maternal side.

3. Preparation for documentation

3.1. Place the placenta on a plastic panel with the maternal side facing upwards.

3.2. Expose the maternal side of the placenta to light (uniform distribution using two identical lamps on the left and right sides). Use lamps that have a function for adjusting light intensity.

3.3. To reduce reflection, take digital photos with a camera (**Table of Materials**) in mild light conditions and avoid direct exposure to the light source.

3.4. Take photos perpendicular to the maternal side of placenta.

4. Computerized measurement using ImageJ

4.1. Open ImageJ on the computer by clicking on the software icon.

4.2. Click on "**File**", then "**Open**" to open the placental picture. Once the required image is opened, click on "**Image**" **|** "**Color**", then "**Channels Tool**" to choose the "**Color**" from the drop list.

NOTE: The software can automatically identify the color of the object measured. The placenta is red. In the software, after clicking "**Color**", the placental picture will automatically be converted into a red spectrum channel.

4.3. Click on "**Freehand selections**" to respectively contour the plethoric and pale placental share of the polycythemic and anemic twins via visual inspection of different color intensity of the maternal side of the placenta.

4.4. Click on "**Analyze**" **|** "**Histogram**". Obtain the color intensity histogram after selecting the plethoric area or pale area, respectively. The mode displayed in the intensity histogram represents the color that is most present in the placental area selected.

4.5. Calculate the color difference ratio by dividing the mode of color intensity of the anemic twin by the mode of color intensity of the polycythemic twin.

REPRESENTATIVE RESULTS:

Placental examination provides valuable information for the diagnosis of TAPS. TAPS placentas are characterized by the presence of few small vascular anastomoses[1] (**Figure 1, Figure 2**). This feature of TAPS placentas is related to the pathogenesis of TAPS and is a postnatal diagnostic criterion. Similar to the color of neonates at birth, the placenta share of the recipient twin in TAPS is typically dark and plethoric, and the placenta share of the donor twin is pale and anemic (**Figure 3**).

The results show that the measurement of color difference between two placental territories on maternal side is feasible using ImageJ (**Figure 4, Figure 5**). In the TAPS placenta exemplified in this study, the mode of color intensity histogram in polycythemic and anemic areas is 82 and 209, respectively, yielding a color difference ratio of 2.5 (209/82) (**Figure 6**). All human monochorionic twin placentas are postnatally examined at the Leiden University Medical Center as a part of standard care for monochorionic twins over the past 25 years. More than 200 TAPS placentas have been examined to confirm the effectiveness of this method.

FIGURE LEGENDS:

Figure 1: Characteristics of spontaneous TAPS placentas. Fetal side of a spontaneous TAPS placenta after color dye injection showing the typical angioarchitecture with only two small

arteriovenous (AV) anastomoses (white arrows) and one small arterio-arterial (AA) anastomosis (blue arrow).

Figure 2: Characteristics of post-laser TAPS placentas. A post-laser TAPS placenta after color dye injection demonstrating two miniscule residual AV anastomoses. The white, dotted line shows the laser line. The area where the residual anastomoses are localized is enlarged in the inserted picture. The two residual miniscule AV anastomoses are indicated by white arrows.

Figure 3: Color difference in TAPS placentas. Maternal side of a spontaneous TAPS placenta showing the striking color difference between the two placental shares.

Figure 4: Conversion of color spectrum in placental pictures. Procedure to convert the digital placental picture into a red spectrum channel in ImageJ.

Figure 5: Analysis of the color intensity in each placental share. Procedure to determine the mode of color intensity histogram of each placental share.

Figure 6: Measurement of color difference ratio in a spontaneous TAPS placenta. The mode of color intensity histogram of each placental share is shown in the red rectangle. The mode of color intensity histogram in the anemic and polycythemic placental shares is 209 and 82, respectively, yielding a color difference ratio of 2.5 (209/82).

DISCUSSION:

Key points to achieving an optimal examination based on our experience include: 1) delivery of the placenta as gently as possible to avoid damage, especially when manual removal is indicated; 2) examination of MC placentas soon after birth to avoid the formation of adhesive clots (preferably within a few days); 3) gentle removal of all blood clots, avoiding damage to the placental lobes; 4) exposure of the maternal side of MC placentas to uniform light conditions and minimization of the light reflection; and 5) taking pictures of the maternal side before dye injection of the fetal side, then quantification of color difference ratio using ImageJ. In this protocol, the ImageJ was used to determine the color difference. Other softwires with function to quantify color intensity are appropriate alternatives. The advantage of ImageJ is its wide application in scientific research and free access. The main limitation of this protocol is the difficulty in setting the identical light intensity and angle for placental pictures. Therefore, it is strongly recommended to use lamps with adjustable light intensity.

All monochorionic placentas have vascular anastomoses connecting the two fetal circulations. These anastomoses may lead to complications such as twin-twin transfusion syndrome (TTTS) or TAPS[7-10]. TTTS may occur in 10% of monochorionic twins and is characterized by a large difference in amniotic fluid with oligohydramnios in the donor twin and polyhydramnios in the recipient twin[11]. TAPS is a newly reported complication that may occur spontaneously in 5% of monochorionic twins or in 13% of TTTS treated with fetoscopic laser surgery. TAPS is characterized by a large inter-twin Hb difference without the degree of amniotic fluid

discordances required for the diagnosis of TTTS[1,5,12]. Prenatal diagnosis of TAPS is currently based on discordant measurements of the middle cerebral artery peak systolic velocity (MCA-PSV) (>1.5 multiples of the median [MoM] in donors and <1.0 in recipients) or a delta MCA-PSV of >0.5 MoM using doppler ultrasound. Postnatal diagnosis of TAPS is based on the presence of large Hb difference (>8 g/dL) and at least one of the following: reticulocyte count ratio (>1.7) or miniscule anastomoses (diameter of <1 mm) detected through colored dye injection[5]. However, complete hematological tests are not always performed at birth, and reticulocyte count measurements are often missing.

In addition, color dye injection is a complex and time-consuming procedure that is not routinely performed in most centers. Search of additional parameters to help confirm a diagnosis of TAPS is warranted. As shown in a recent study, measuring the color difference between two maternal shares of TAPS placentas can be a useful additional criterion. A cut-off value of 1.5 of the color difference ratio (i.e., ratio of the color intensity in the anemic placental share vs. the plethoric placental share) may be helpful to characterize TAPS placentas[6]. The presented protocol provides an accurate and simple method to quantify color differences in the maternal side of TAPS placentas. However, larger studies are required to further evaluate the diagnostic value and determine the cut-off value of color differences of the maternal side for postnatal TAPS diagnosis.

ACKNOWLEDGEMENT:
This study was funded by the National Natural Science Foundation of China (Grant number: 81801465), the Sanming Project of Medicine in Shenzhen (Grant number: SZSM201512012) and Shanghai Pujiang Program (Grant number: 17PJ1407900).

DISCLOSURES:
The authors have nothing to disclose.

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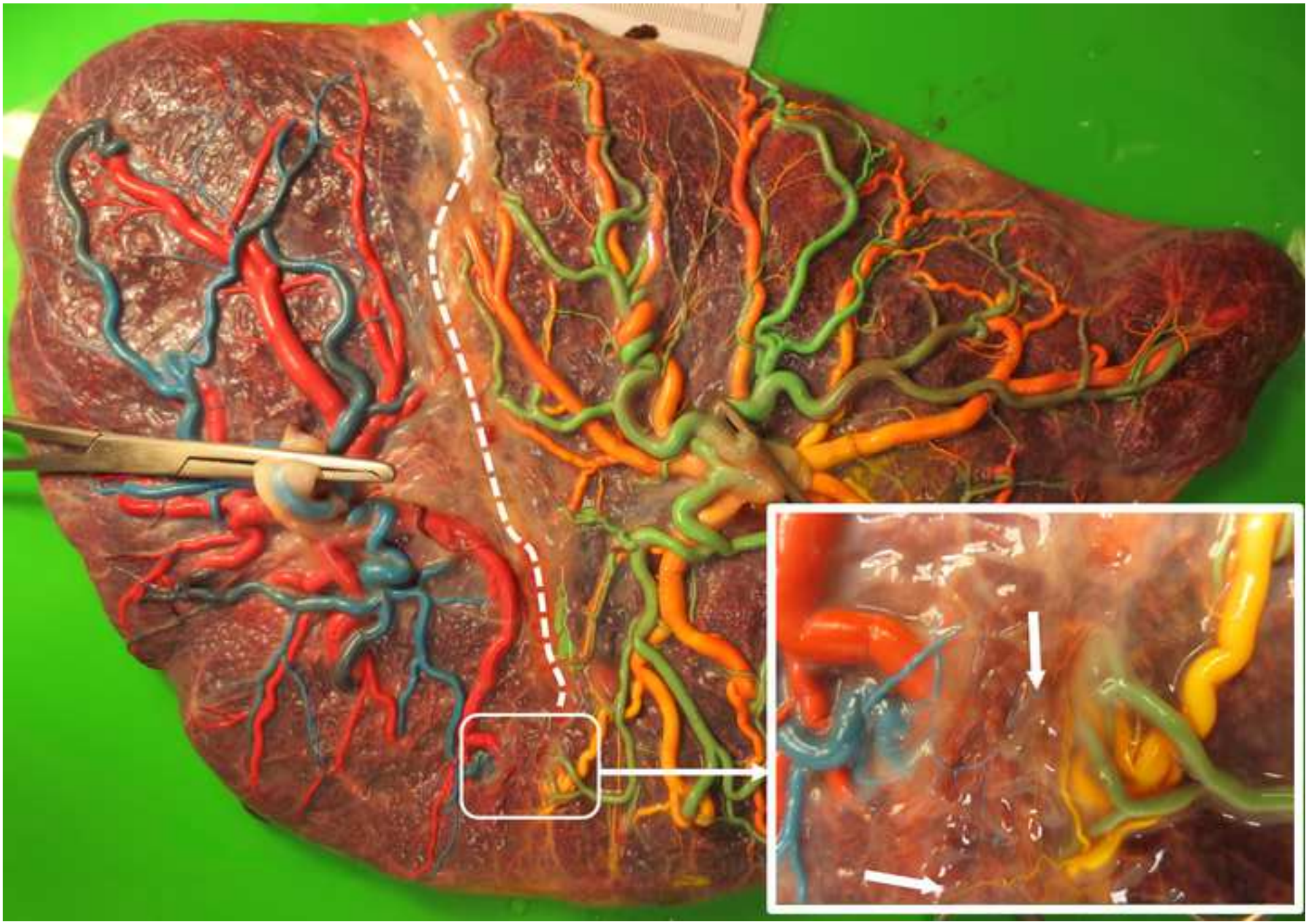
Characteristics of spontaneous TAPS placentas

Click here to access/download;Figure;Figure 1.psd

Characteristics of post-laser TAPS placentas

Click here to access/download;Figure;Figure 2.psd

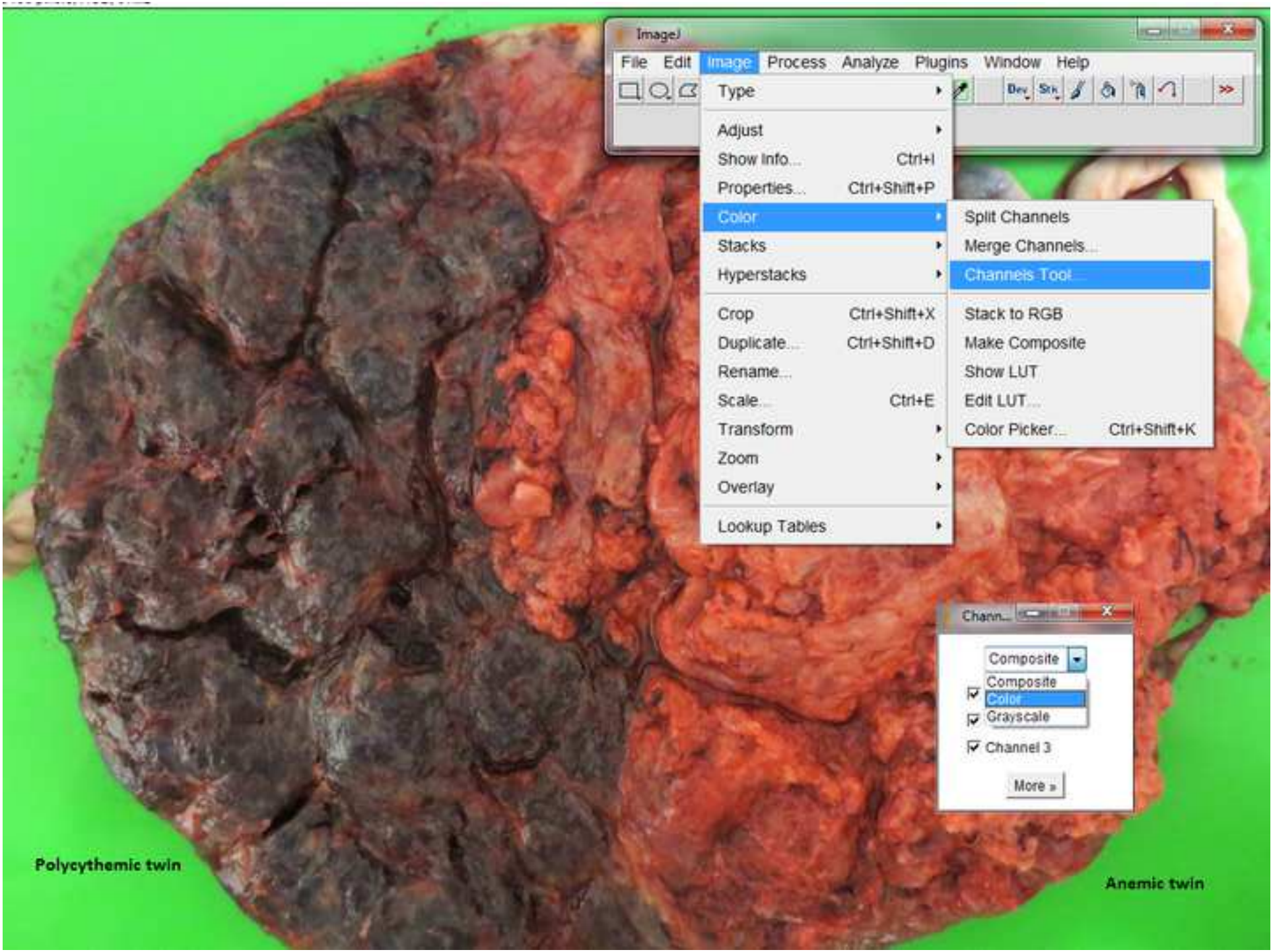


Color difference of TAPS placenta

Click here to access/download;Figure;Figure 3.psd

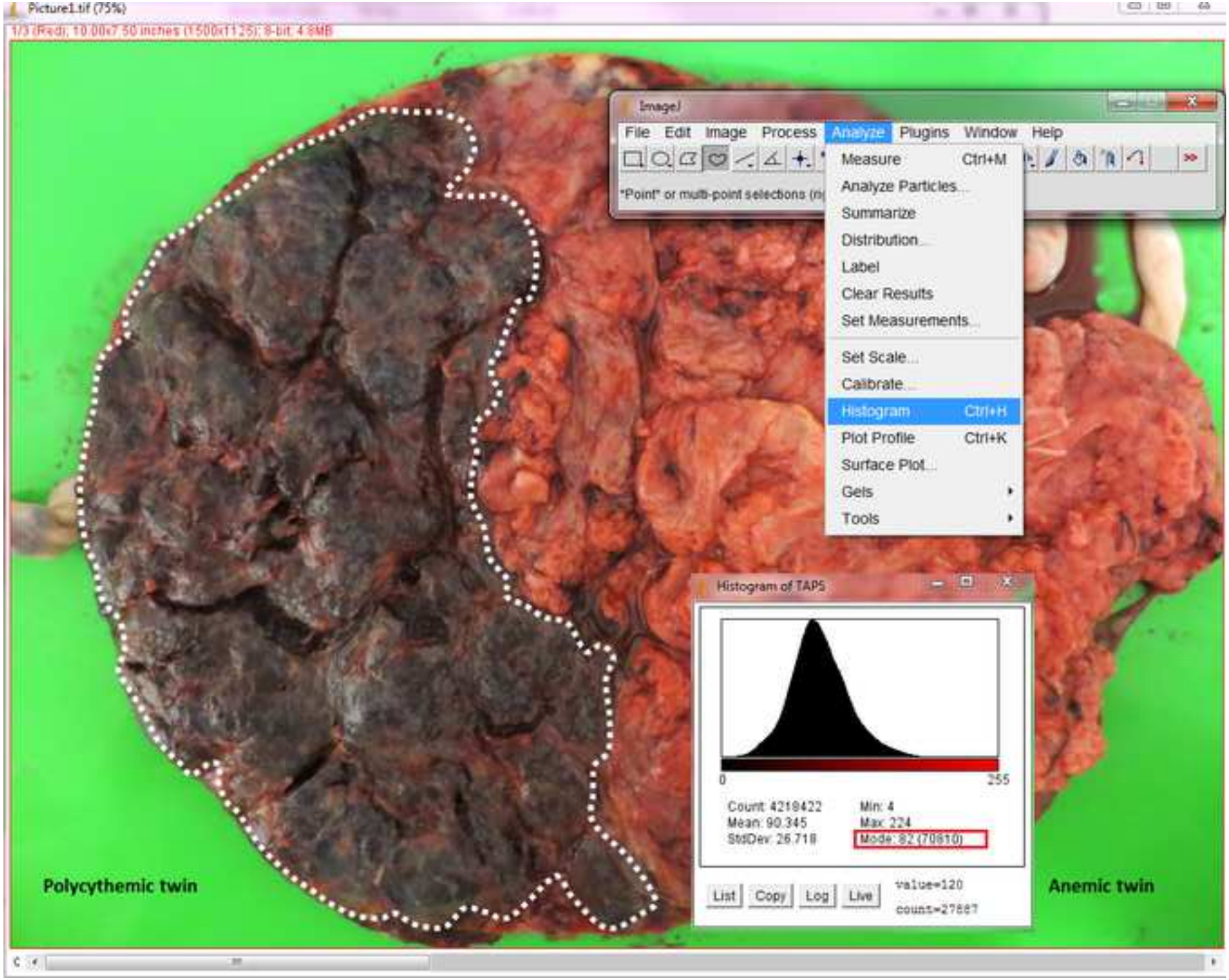
Conversion of color spectrum of placental pictures

Click here to access/download;Figure;Figure 4.psd



Analysis of the color intensity in each placental share

Click here to access/download;Figure;Figure 5.psd



Measurement of the color difference ratio in a spontaneous TAPS placenta

Click here to access/download;Figure;Figure 6.psd

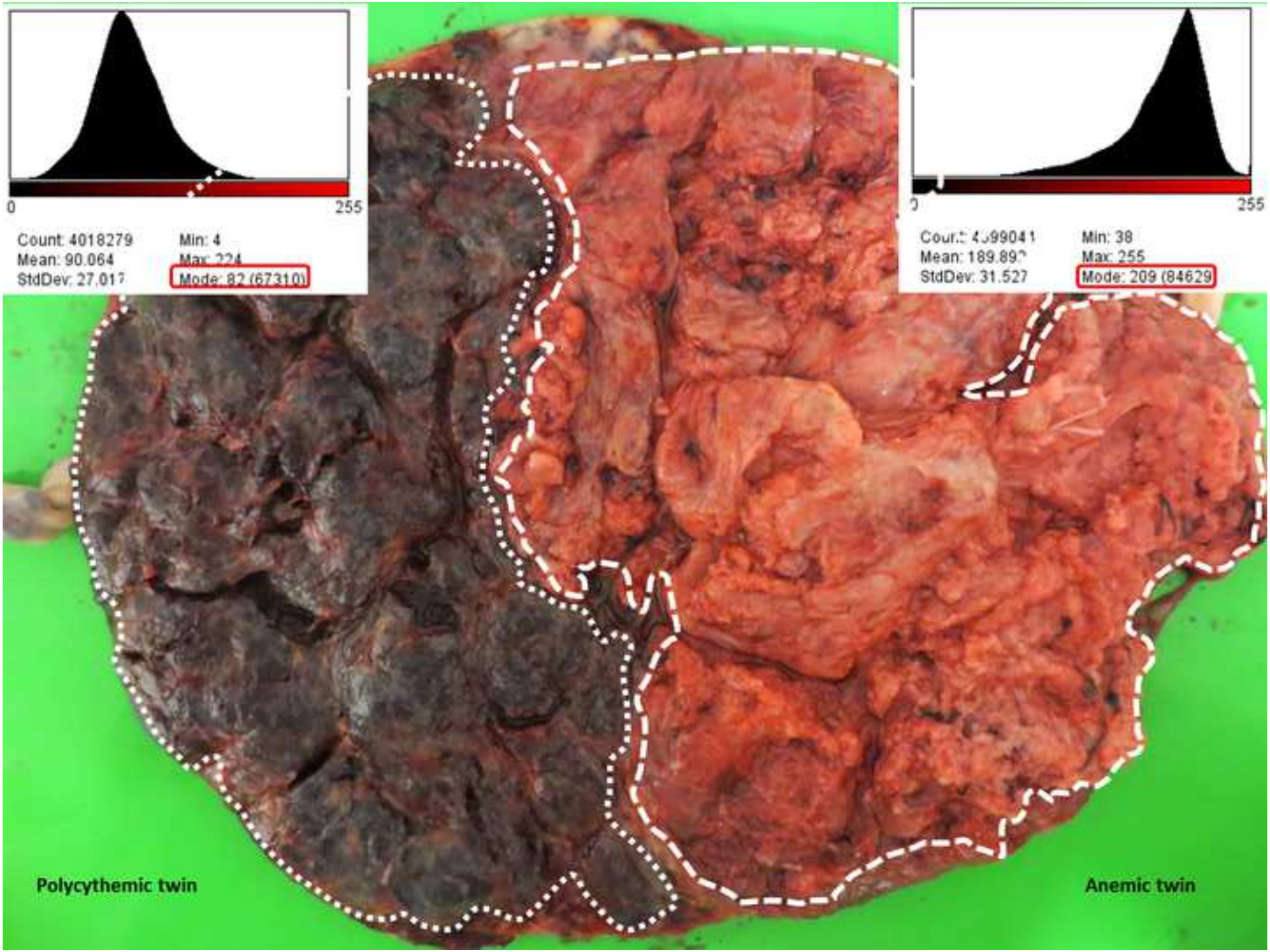


Table of Materials

Name of the reagent	Company	Catalogue number
20 ml syringes	BD Plastipak	300613
Color dye	Royal Talens Schoolverf	36716010 (blue); 36715010 (green); 36712350 (yellow); 36713570 (pink)
Digital camera	Canon Inc.	ixus 125 hs
ImageJ	National Institute of Health	For Windows ImageJ bundled with 64-bit Java 1.8.0_112
Umbilical catheters	Vygon	1270.08 (8 F)
		1270.08 (5 F)
		1270.04 (4 F)
		1270.03 (3.5 F)
		1270.02 (2.5 F)

Dear Editor,

We thank you for the meaningful comments of the reviewers and appreciate the opportunity to revise our manuscript. We were able to adapt our paper according to the suggestions of the reviewers.

1. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi.

Reply: All the figures were saved as the .psd style.

2. Additionally, please upload tables as .xlsx files.

Reply: Table 1 was uploaded as .xlsx file.

Editorial comments:

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.

Rely: All the authors did the proofreading of this manuscript before resubmission.

2. Please provide at least 6 keywords or phrases.

Reply: 6 keywords or phrases.were provided (Please see Page 2 Lines 17-18).

3. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Presented here is a protocol …”

Reply: The Summary was added as “Presented here is a protocol to to quantify the

color difference in maternal side of TAPS placentas after birth." (Please see Page 3, Lines 5-6)

4. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

Reply: The word count of the long Abstract is 244. The goal of this protocol was clearly stated (Please see Page 4, Lines 2-4).

5. Please expand the Introduction to include all of the following with citations:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

Reply: The Introduction section was added according to the above guidance. (Pease see Page 4 Lines 5-15)

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Reply: The ethics statement was added. (Please see Page 4 Lines 16-18)

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The

actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Reply: The phase such as “could be,” “should be,” and “would be” were rephrased and the imperative tense was used throughout the Protocol.

8. The Protocol should contain only action items that direct the reader to do something.

Reply: The protocol was revised according to the guidance.

9. Please ensure you answer the “how” question, i.e., how is the step performed? Please include all the button clicks in the software, knob turns etc. Please use complete sentences to describe the action. e.g., Click on “Open” to open the files, then click “Analyze”

Reply: the protocol was revised accordingly.

10. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Reply: The essential steps for the video were highlighted as yellow.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited

appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Reply: The placental pictures used in this protocol were not publised elsewhere.

12. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

Reply: The Figure Legend section was revised.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraph style with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Reply: The Discussion section was revised as suggested (Page 9 Lines 19-21, Page 10 Lines 1-2).

14. Please do not abbreviate the journal titles in the reference section.

Reply: The reference format was revised.

15. Figures: Please include a scale bar and describe it in the figure legend.

Reply: A ruler was put next to the placenta when the picture was taken (Please see Figure 1, the ruler was put on the top). Therefore, the ruler functions as the scale bar in the analysis by ImageJ.

16. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials

in separate columns. Please sort the table in alphabetical order.

Reply: The information required was provided.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript entitled "Evaluation of color difference in placentas with twin anemia-polycythemia sequence" is demonstrating the protocol for quantifying the color difference in TAPS placentas. This manuscript is overall well written and demonstrates interesting ideas and the authors have described the precise and detail protocol which seems to be replicable easily. Therefore, I conceive that the protocol described in this document will be quite useful for the readers of JoVE after minor revision listed on the comments.

Minor Concerns:

Please mention that the classification system for TAPS has been updated last year. Instead of cut-off values for anemia (>1.5 MoM) and polycythemia (<1.0 MoM), a delta MCA-PSV > 0.5 MoM could be also used to diagnose TAPS. Please add the reference "Improved Prediction of Twin Anemia-Polycythemia Sequence by Delta Middle Cerebral Artery Peak Systolic Velocity: New Antenatal Classification System" Ultrasound Obstet Gynecology, 2019.

Reply: Thank you for this comment. This publication was added in the Reference.

Reviewer #2:

Manuscript Summary:

This is a protocol for placental examination in monochorionic twin pregnancy complicated by twin anemia polycythemia sequence: it is very well written and thought, feasible and reproducible.

The importance of a standardized protocol for monochorionic placental examination lies in the need of investigate further causes of monochorionic twin complication. In this specific case, TAPS is the last challenge for researchers, since a proven cause has not been found at all. This could be a starting point for collaborative multi-center study of TAPS twins.

Major Concerns:

The authors state that TAPS is the consequence of the imbalance blood flow through a little arterovenous anastomosis: since it has not been demonstrated this is the only etiology, they should better write that this is the main pathogenetic hypothesis.

Reply: We agree with this comment. The sentence was rephrased (Please see Page 3 Line 10).

The authors use ImageJ software for pics analysis: in case other researchers are more confident with other software, they should be allowed to use them unless they reach the same results.

Reply: Thank you for this comment. This suggestion was added in the Discussion

section (Please see Page 9 Lines 19-21).

We hope our changes in the manuscript will satisfy the reviewers. Thank you for your consideration for publishing our manuscript in your journal.

Kind Regards

Depeng Zhao