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Corresponding Author:	Na Li School of Medicine, Nankai University Tianjin, Tianjin CHINA
Corresponding Author's Institution:	School of Medicine, Nankai University
Corresponding Author E-Mail:	lina08@nankai.edu.cn
Order of Authors:	Xiaoli Dong
	Yingying Wei
	Tao Xu
	Xiaoyue Tan
	Na Li
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TITLE:

Analysis of Side Population in Solid Tumor Cell Lines

AUTHORS AND AFFILIATIONS:

Xiaoli Dong[1], Yingying Wei[1], Tao Xu[1], Xiaoyue Tan[1,2,3], Na Li[1,2,3]

[1]School of Medicine, Nankai University, 94 Weijin Road, Tianjin, China
[2]Tianjin Key Laboratory of Tumour Microenvironment and Neurovascular Regulation, Tianjin, China
[3]Collaborative Innovation Center for Biotherapy, Nankai University, 94 Weijin Road, Tianjin, China

Corresponding Authors:

Xiaoyue Tan (xiaoyuetan@nankai.edu.cn)
Na Li (lina08@nankai.edu.cn)

Email Addresses of Co-authors:

Xiaoli Dong (dongxiaolidxl@163.com)
Yingying Wei (1528021668@qq.com)
Tao Xu (978565285@qq.com)

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SUMMARY:

A convenient, fast, and cost-effective method to measure the proportion of side population cells in solid tumor cell lines is presented.

ABSTRACT:

Cancer stem cells (CSCs) are an important cause of tumor growth, metastasis, and recurrence. Isolation and identification of CSCs are of great significance for tumor research. Currently, several techniques are used for the identification and purification of CSCs from tumor tissues and tumor cell lines. Separation and analysis of side population (SP) cells are two of the commonly used methods. The methods rely on the ability of CSCs to rapidly expel fluorescent dyes, such as Hoechst 33342. The efflux of the dye is associated with the ATP-binding cassette (ABC) transporters and can be inhibited by ABC transporter inhibitors. Methods for staining cultured tumor cells with Hoechst 33342 and analyzing the proportion of their SP cells by flow cytometry are described. This assay is convenient, fast, and cost-effective. Data generated in this assay can contribute to a better understanding of the effect of genes or other extracellular and intracellular signals on the stemness properties of tumor cells.

INTRODUCTION

Cancer stem cells (CSCs) are subsets of cells with self-renewal ability and multiple differentiation potential, which play a vital role in tumor growth, metastasis, and recurrence[1,2]. Currently, CSCs have been identified to exist in a variety of malignant tumors, including lung, brain, pancreas, prostate, breast, and liver cancers[3–9]. Identification of CSCs in these tumors is mainly based on the presence of surface marker proteins, such as high and/or low expression of CD44, CD24, CD133, and Sca-1[9,10], but a unique marker that can distinguish CSCs from non-CSCs has not been reported so far. Currently, several techniques are used to identify and purify CSCs in tumor tissue or tumor cell lines. These techniques are designed based on the specific properties of CSCs. Among them, assays and sorting of side population (SP) cells are two of the commonly used methods.

SP cells were originally discovered by Goodell et al.[11], when they characterized hematopoietic stem cells in mouse bone marrow cells. When the mouse bone marrow cells were labeled with the fluorescent dye Hoechst 33342, a small group of Hoechst 33342 low-stained cells appeared in the two-dimensional dot plot of a flow cytometry assay. Hoechst 33342 is a DNA-binding dye that emits both blue and red fluorescence under UV excitation. Hoechst 33342 has at least two binding modes that lead to different spectral characteristics. When viewing fluorescence emission at two wavelengths at the same time, multiple populations can be revealed[12]. In this assay, the Hoechst 33342 was excited at 350 nm and the fluorescence was measured under a two-wavelength filter (450/20 nm band-pass [BP] filter and 675 nm edge filter long-pass [EFLP])[11]. Compared with whole population of bone marrow cells, this group of cells was enriched with hematopoietic stem cells called SP cells[11]. SP cells are capable of rapidly expelling Hoechst 33342. The efflux of this dye is related to ABC transporters[13], which can be inhibited by some agents such as Fumitremorgin C[14] and calcium channel inhibitors, including Verapamil and Reserpine[15,16]. After that, different proportions of SP cells were detected in a variety of tissues, organs, tumor tissues, and tumor cell lines[17–19]. These SP cells have many characteristics of stem cells[17,19].

This manuscript describes Hoechst 33342 labeling and staining of cultured tumor cells and the analysis of SP cells by flow cytometry. Moreover, the effects of stemness promotion or inhibition signals on the proportion of SP in tumor cells are demonstrated. Finally, optimization of the Hoechst 33342 concentration and the proper blocker selection for a specific tumor cell line using this approach are discussed. The experimental examples shown demonstrate that analysis of SP can be used to explore the effects of various signals, such as gene expression, small inhibitors, activators, cytokines, and chemokines on tumor stemness. Compared to other methods for isolation and purification of CSCs, such as sorting of $CD44^+/CD24^-$ population, aldehyde dehydrogenase (ALDH) analysis, and tumor sphere formation assays, this method is easier for manipulation and is cost-effective.

PROTOCOL

1. Cell preparation

1.1. Cell digestion and neutralization

1.1.1. Seed tumor cells (such as MDA-MB-231 cells) in a 6 well plate, and culture them in a 37 °C incubator supplied with 5% CO_2.

1.1.2. Harvest cells when their density reaches 90%. Aspirate the culture medium thoroughly and wash the cells 2x with 3 mL of phosphate buffered saline (PBS).

NOTE: To examine the effects of signaling pathway inhibitors (e.g., FRA1 inhibitor), or activators (e.g., STAT3 activator) on stemness features of tumor cells, tumor cells were seeded in a 6 well plate and pretreated with inhibitors or activators for a specific number of hours before harvest.

1.1.3. Add 500 µL of 0.25% trypsin-EDTA to the 6 well plate, place the plate in an incubator at 37 °C for 1–3 min, and gently tap the plate to detach the cells.

NOTE: Prolonged digestion will affect the SP profile due to changes in cell viability.

1.1.4. Add 3 mL of PBS supplemented with 2% FBS to terminate the digestion and gently pipette the cells up and down 3–5x to disperse any cell clumps.

1.1.5. Add 0.5 mL of cell suspension to one well of a new 6 well plate and examine it under a microscope to verify the existence of single cells. If cell clumps are observed, pass the cell suspension through a 70 µM cell strainer.

NOTE: This is an optional step.

1.1.6. Transfer the contents to a new 15 mL centrifuge tube. Centrifuge cells at 200 x *g* for 5 min to pellet cells. Remove the supernatant and resuspend them in 3 mL of PBS supplemented with 2% fetal bovine serum (FBS). Pipette the cells up and down 3–5x to mix thoroughly.

1.2. Cell counts

1.2.1. Add 50 µL of the cell suspension into a 1.5 mL microcentrifuge tube and mix it with 50 µL trypan blue solution. Pipette 10 µL of the mixture to count the number of living cells using a standard method, such as a hemocytometer.

1.2.2. Dilute the cells in PBS supplemented with 2% FBS to a final concentration of 1 x 10^6 cells/mL. Add 1 mL of the cell suspension to a 5 mL polystyrene round bottom test tube and prepare two sample tubes.

2. Cell staining with Hoechst 33342

2.1. Add Hoechst 33342 to one tube to reach an appropriate final concentration.

NOTE: For example, the appropriate concentration of Hoechst 33342 is 3 µg/mL for MDA-MB-231 cells.

2.2. Add blockers (e.g., Fumitremorgin C, Verapamil, or Reserpine) to another tube to an appropriate final concentration and incubate the tube at 37 °C for 30 min before adding the same concentration of Hoechst 33342 as described in step 2.1.

NOTE: For MDA-MB-231 cells, the appropriate blocker is Reserpine (40 µM). Reserpine is used as a blocking control to verify the absence of cells in the gated SP area.

2.3. Prepare several tubes containing cells and add Hoechst 33342 with different concentration gradients. After a flow cytometry assay, choose the proper concentration according to the profile and proportion of SP.

NOTE: This is an optional step used to define the proper concentration of Hoechst 33342.

2.4. Prepare several tubes containing cells, add the different concentration gradients of the blocker, incubate the tubes at 37 °C for 30 min, then add Hoechst 33342 to an appropriate concentration. After the flow cytometry assay, choose the proper concentration according to the absence of cells in the gated SP area.

NOTE: This is an optional step used to define the proper concentration of blocker.

2.5. Place the tubes in a 37 °C incubator and incubate them for 60 min, shaking the tubes every 10 min.

NOTE: Shaking tubes thoroughly is important for staining, because it ensures complete contact between the cells and the dye for better staining results.

2.6. After 60 min, centrifuge the cells at 200 x *g*, 4 °C for 5 min, and aspirate the supernatant carefully, because the cells form a very loose and unstable pellet.

2.7. Resuspend cells in 1 mL of ice-cold PBS supplemented with 2% FBS and pipette the cells up and down 3–5x to mix thoroughly. Add 1 µL of 1 mg/mL propidium iodide (PI) to the suspension to identify dead cells.

NOTE: All procedures in this step should be performed at 4 °C to inhibit the efflux of Hoechst 33342 from the tumor cells. The tubes should be protected from direct exposure to light.

3. Analysis by flow cytometry

NOTE: Instructions for use of the flow cytometer software (see **Table of Materials**) are

described in this section and **Supplementary Figures 1–10**.

3.1. In the flow cytometer software, click the **FOLDER** button. Click the **Experiment** button, then click **New Experiment** (**Supplementary Figure 1A**,**B**).

3.2. Click the **OK** button. “Experiment-001” will show up under the **FOLDER** (**Supplementary Figure 2A**,**B**).

3.3. Click the **Experiment-001** button to change the folder name to a specific name (e.g., "20191118-SP"). Click **Enter**. The new name ("20191118-SP") will show up under **FOLDER** (**Supplementary Figure 3A**,**B**).

3.4. Click the **New Specimen** button to add a specimen to the new experiment folder ("20191118-SP"). Click the **New Tube** button to add a tube to the specimen. Click the **Arrowhead** button (**Supplementary Figure 4A**,**C**).

3.5. Click the **Parameters** button and set up the parameters of the flow cytometer (**Supplementary Figure 5**).

3.5.1. Use a 610 nm dichroic mirror short pass (DMSP) to separate the emission wavelengths. Use a 450/20 nm BP filter to collect the blue fluorescence and a 675 nm EFLP to collect the red fluorescence.

NOTE: Hoechst 33342 is excited with a UV laser at 355 nm and PI is excited at 488 nm.

3.6. Run the cell samples on the flow cytometer.

NOTE: SP cells can be sorted by fluorescence activated cell sorting (FACS) under sterile conditions. A total of 100,000–500,000 cells should be collected for the follow-up experiment.

3.6.1. Run cells stained with Hoechst 33342 on the flow cytometer.

3.6.1.1. Place tubes containing cells stained with Hoechst 33342 on the cytometer.

3.6.1.2. Click the **Dot Plot** button to display the dot plot, then click the **X-axis** and set it to “**FSC-A**”; click the **Y-axis** and set it to “**PI-A**”. Display the dot plot of forward scatter pulse area (FSC-A, X-axis set to linear mode) versus the PI fluorescence (Y-axis set to logarithmic scale). Adjust the voltages to show the living cells in the right side and the non-living cells, which are strongly stained with PI, in the left higher corner. Then, establish a polygon gate to exclude dead cells and cell debris (**Figure 1A**) by clicking the **Polygon Gate** button to gate the P1 subset, also known as FSC-A, PI-A subset (**Supplementary Figure 6A**,**B**).

3.6.1.3. Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to “**FSC-A**”;

click the Y-axis and set it to "**FSC-W**". Display the dot plot of FSC-A (X-axis) versus forward scatter pulse width (FSC-W, Y-axis). Right-click the **dot plot**, click the **P1** button under the **Show Populations** button. Click the **Rectangular Gate** button to create a rectangular gate to gate the P2 subset (also known as FSC-A, FSC-W subset). This will exclude cells with large volumes (**Supplementary Figure 7A**,**C** and **Figure 1A**).

3.6.1.4. Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to "**SSC-A**"; click the **Y-axis** and set it to "**SSC-W**". Display the dot plot of side scatter pulse area (SSC-A, X-axis) versus side scatter pulse width (SSC-W, Y-axis). Right-click the **dot plot**, and click the **P2** button under **Show Populations** button. Then click the **Rectangular Gate** button to create a rectangular gate to gate the P3 subset (also known as SSC-A, SSC-W subset). This will obtain a cell population with uniform granularity (**Supplementary Figure 8A**,**C** and **Figure 1A**).

3.6.1.5. Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to "**Hoechst Red-A**"; click the **Y-axis** and set it to "**Hoechst Blue-A**". Display the dot plot of Hoechst Red-A (X-axis) versus Hoechst Blue-A (Y-axis). Right-click the **dot plot**, click the **P3** button under the **Show Populations** button. Click the **Polygon Gate** button to create a polygon gate to gate the P4 subset (also known as Hoechst Red-A, Hoechst Blue-A subset). Right-click the **dot plot**, click the **Show Population Hierarchy** button to the show population hierarchy.

NOTE: The dot plot will show three different populations: 1) a G0-G1 phase population near the center of the graph; 2) a S-G2/M phase population near the upper right corner; 3) the SP. The SP is then gated for further analysis (**Supplementary Figure 9A**,**D** and **Figure 1A**). If the dot plot of Hoechst Red-A versus Hoechst Blue-A does not show an SP profile similar to that shown in **Figure 1A**, the voltages should be adjusted until a similar profile is seen. Meanwhile, adjust all of above gates accordingly.

3.6.1.6. After determining the gate region of the SP cells, click **Acquire Data** to collect 20,000–100,000 events from each sample to analyze the percentage of SP cells (**Supplementary Figure 10**).

3.6.2. Run the Hoechst 33342-stained cells treated with blocker on the flow cytometer.

3.6.2.1. Place tubes containing blocker on the cytometer and run cells using the same voltages and gates to further check whether the voltages and gates are selected appropriately.

NOTE: Only a very small proportion of cells, if any, should appear in the gated area of the SP compared with Hoechst 33342 staining alone (**Figure 1B**).

3.6.2.2. Collect 20,000–100,000 events from each sample to analyze the percentage of SP cells.

4. Data analysis

NOTE: Instructions for the use of the flow cytometry analysis software (see **Table of Materials**) are described in this section and **Supplementary Figures 11–16.**

4.1. Copy the files in fcs format to a computer, open the flow cytometry analysis software, and drag one sample file to the software (**Supplementary Figure 11A**,**B**)**.**

4.2. Gate cells and obtain the percentage of SP cells.

4.2.1. Double click this **sample file**, click the **X-axis** and set it to "**FSC-A**"; click the **Y-axis** and set it to "**PI-A**". Then, create a polygon gate and click the **OK** button to obtain the FSC-A, PI-A subset (**Supplementary Figure 12A**,**E**).

4.2.2. Double click the **FSC-A, PI-A subset file**, click the **X-axis** and set it to "**FSC-A**"; click the **Y-axis** and set it to "**FSC-W**". Then, create a rectangular gate and click the **OK** button to obtain the FSC-A, FSC-W subset (**Supplementary Figure 13A**,**E**).

4.2.3. Double click the **FSC-A, FSC-W subset file**, click the **X-axis** and set it to "**SSC-A**"; click the **Y-axis** and set it to "**SSC-W**". Then, create a rectangular gate and click the **OK** button to obtain the SSC-A, SSC-W subset (**Supplementary Figure 14A**,**E**).

4.2.4. Double click the **SSC-A, SSC-W subset file**, click the **X-axis** and set it to "**Hoechst Red-A**"; click the **Y-axis** and set it to "**Hoechst Blue-A**". Then, create a polygon gate and click the **OK** button to obtain the Hoechst Red-A, Hoechst Blue-A subset (**Supplementary Figure 15A**,**E**).

4.2.5. Open the Layout Editor by clicking the **Open Layout Editor** button. Drag the SSC-A, SSC-W subset sample file to Layout Editor, then click the **Click to save layout window to file** button to save the image results (**Supplementary Figure 16A**,**C**).

4.3. Save the workspace to keep the gating information when closing the software.

4.4. Perform t-test analyses with statistical analysis software to compare the difference between the two groups. A value of $P < 0.05$ was defined as statistically significant.

REPRESENTATIVE RESULTS:

Four experimental SP analyses were performed according to this method. In the first one, we detected the proportion of SP cells in MDA-MB-231, which is a triple negative human breast cancer cell line, under normal conditions. After cell counting, Hoechst 33342 was added into one tube containing 1 x 10^6 cells to a final concentration of 3 μg/mL. Reserpine and Hoechst 33342 were added to another tube to a final concentration of 40 μM and 3 μg/mL, respectively. PI was added to both tubes. The dot plot of FSC-A (X-axis) versus PI-A

(Y-axis) showed three populations: 1) a PI-positive cell population, which represents the dead cells; 2) cell debris; and 3) the main population was PI-negative cells, which were subjected to further analysis (**Figure 1A**,**B**). A single-cell population gated from the dot plot of FSC-A (X-axis) versus FSC-W (Y-axis) and the dot plot of SSC-A (X-axis) versus SSC-W (Y-axis) was used to analyze the proportion of SP cells (**Figure 1A**,**B**). The SP cells were gated from the dot plot of Hoechst Red-A (X-axis) versus Hoechst Blue-A (Y-axis), and its percentage was about 0.9% in MDA-MB-231 cells (**Figure 1A**). However, Reserpine significantly decreased the proportion of SP cells (**Figure 1B**), supporting that the gate-painting for SP is correct. In addition, the dot plot of Hoechst Red-A (X-axis) versus Hoechst Blue-A (Y-axis) showed the population of cells in G0-G1 phase and S-G2/M phase (**Figure 1A**,**B**).

The second experiment was to determine the suitable staining concentration of Hoechst 33342 in MDA-MB-435 cells. After cell counting, Hoechst 33342 was added to 1 x 10^6 cells suspended in 1 mL of PBS supplemented with 2% FBS at different concentration gradients, including 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 μg/mL. As shown in **Figure 2A**, when the concentration of Hoechst 33342 was too low (i.e., 0.5, 1, 1.5 μg/mL), it was hard to distinguish SP cells from other cell populations, because lots of cells were in a low-stained state. When the concentration of Hoechst 33342 was too high (i.e., 2.5, 3, 3.5, 4 μg/mL), SP cells decreased until they disappeared, and the profile changed greatly. Thus, 2 μg/mL Hoechst 33342 was the best concentration for SP analysis in MDA-MB-435 cells. In addition, to determine the proper blocker for this cell line, Hoechst 33342 and a blocker (Verapamil or Reserpine) were added to 1 x 10^6 cells suspended in 1 mL of PBS supplemented with 2% FBS, to a final concentration of 2 μg/mL and 40 μM, respectively. As shown in **Figure 2B**, about 0.4% of cells expelled the dye after Verapamil treatment. However, after Reserpine treatment, the ratio dropped to about 0.1% (**Figure 2C**). From this experiment, Reserpine was considered a more appropriate blocker for this cell line.

In the third example, A549 cells (human lung adenocarcinoma cells) were pretreated with STAT3 activator-Colivelin[20] (100 nM) for 48 h. The STAT3 signaling pathway is important for promoting the stemness features of tumor cells[21]. As shown in **Figure 3**, the proportion of SP cells increased upon Colivelin stimulation.

In the last example, T47D cells (human breast cancer cells) were pretreated with 0.1 μM FRA1 inhibitor-SKLB816 (also named 13an) for 48 h[22]. FRA1 is a reported gatekeeper of mesenchymal-epithelial transition (EMT) and involved in regulation of tumor stemness[23]. Cells were harvested, counted, and stained with Hoechst 33342. As shown in **Figure 4**, the proportion of SP cells decreased due to the FRA1 inhibitor.

FIGURE LEGENDS:

Figure 1: Gating strategy of MDA-MB-231 cells in SP analysis. (**A**) Gating strategy of MDA-MB-231 cells stained with Hoechst 33342 (3 μg/mL) and propidium iodide (PI, 1 μg/mL). (**B**) Gating strategy of MDA-MB-231 cells treated with Reserpine (40 μM), stained with Hoechst 33342 (3 μg/mL) and PI (1 μg/mL).

Figure 2: Optimization of Hoechst 33342 concentration and selection of blocker in MDA-MB-435 cells. (**A**) MDA-MB-435 cells stained with Hoechst 33342 at different concentration gradients (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 μg/mL) together with PI (1 μg/mL). (**B**) MDA-MB-435 cells treated with Verapamil (40 μM) stained with Hoechst 33342 (2 μg/mL) and PI (1 μg/mL). (**C**) MDA-MB-435 cells treated with Reserpine (40 μM) stained with Hoechst 33342 (2 μg/mL) and PI (1 μg/mL).

Figure 3: The proportion of SP cells was enhanced by STAT3 activator in A549 cells. (**A**) A549 cells pretreated with STAT3 activator Colivelin (100 nM) or its vehicle control (Ctrl)-H_2O for 48 h stained with Hoechst 33342 (7 μg/mL) and PI (1 μg/mL). A549 cells treated with Reserpine (45 μM) were used as the blocking control. (**B**) The statistical results of the proportion of SP cells in A549 cells treated with Colivelin (100 nM) and its vehicle control. Data are presented as mean + standard error of the mean (SEM), n = 3 for each group. "*" indicates $P < 0.05$.

Figure 4: The proportion of SP cells was inhibited by FRA1 inhibitor in T47D cells. (**A**) T47D cells pretreated with SKLB816 (0.1 μM) or its vehicle control (Ctrl)-DMSO for 48 h stained with Hoechst 33342 (8 μg/mL) and PI (1 μg/mL). T47D cells treated with Reserpine (40 μM) were used as the blocking control. (**B**) The statistical results of the proportion of SP cells in T47D cells treated with SKLB816 (0.1 μM) and its vehicle control. Data are presented as mean + SEM, n =3 for each group. "*" indicates $P < 0.05$.

Supplementary Figure 1: Instructions for flow cytometer software step number 3.1. (**A**) Click the **FOLDER** button. (**B**) Click the **Experiment** button and then click the **New Experiment** button.

Supplementary Figure 2: Instructions for flow cytometer software step number 3.2. (**A**) Click the **OK** button. (**B**) **Experiment-001** shows up under **FOLDER**.

Supplementary Figure 3: Instructions for flow cytometer software step number 3.3. (**A**) Click the **Experiment-001** button to change the name of Experiment-001 to a specific name (e.g., "20191118-SP"). (**B**) Click the **Enter** button.

Supplementary Figure 4: Instructions for flow cytometer software step number 3.4. (**A**) Click the **New Specimen** button. (**B**) Click the **New Tube** button. (**C**) Click the **Arrowhead** button.

Supplementary Figure 5: Instructions for flow cytometer software step number 3.5. (**A**) Click the **Parameters** button to set up the parameters.

Supplementary Figure 6: Instructions for flow cytometer software step number 3.6.1.2. (**A**) Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to "**FSC-A**"; click the **Y-axis** and set it to **"PI-A**". (**B**) Click the **Polygon Gate** button to create a polygon gate to

gate the P1 subset (also known as FSC-A, PI-A subset).

Supplementary Figure 7: Instructions for flow cytometer software step number and 3.6.1.3. (**A**) Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to **"FSC-A**"; click the **Y-axis** and set it to **"FSC-W**". (**B**) Right-click the **Dot Plot** and click the **P1** button under **Show Populations** button. (**C**) Click the **Rectangular Gate** button to create a rectangular gate to gate the P2 subset (also known as FSC-A, FSC-W subset).

Supplementary Figure 8: Instructions for flow cytometer software step number 3.6.1.4. (**A**) Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to "**SSC-A**"; click the **Y-axis** and set it to "**SSC-W**". (**B**) Right-click the **Dot Plot** and click the **P2** button under the **Show Populations** button**.** (**C**) Click the **Rectangular Gate** button to create a rectangular gate to gate the P3 subset (also known as SSC-A, SSC-W subset).

Supplementary Figure 9: Instructions for flow cytometer software step number 3.6.1.5. (**A**) Click the **Dot Plot** button to display the dot plot, click the **X-axis** and set it to "**Hoechst Red-A**"; click the **Y-axis** and set it to "**Hoechst Blue-A**". (**B**) Right-click the **Dot Plot** and click the **P3** button under the **Show Populations** button. (**C**) Click the **Polygon Gate** button to create a polygon gate to gate the P4 subset (also known as Hoechst Red-A, Hoechst Blue-A subset). (**D**) Right-click the **Dot Plot**, then click the **Show Population Hierarchy** button to show the population hierarchy.

Supplementary Figure 10: Instructions for flow cytometer software step number 3.6.1.6. (**A**) Click the **Acquire Data** button, then collect 20,000-100,000 events from each sample.

Supplementary Figure 11: Instructions for flow cytometry analysis software step number 4.1. (**A**) Open the flow cytometry analysis software and drag one sample file into the software. (**B**) The sample file is imported**.**

Supplementary Figure 12: Instructions for flow cytometry analysis software step number 4.2.1. (**A**) Double click this **sample file.** (**B**) Click the **X-axis** and set it to "**FSC-A**"; click the **Y-axis** and set it to "**PI-A**". (**C**) Click the **Create a polygon gate** button to create a polygon gate. (**D**) Click the **OK** button to obtain the FSC-A, PI-A subset. (**E**) The FSC-A, PI-A subset is obtained.

Supplementary Figure 13: Instructions for flow cytometry analysis software step number 4.2.2. (**A**) Double click the **FSC-A, PI-A subset file.** (**B**) Click the **X-axis** and set it to "**FSC-A**"; click the **Y-axis** and set it to "**FSC-W**". (**C**) Click the **Create a rectangular gate** button to create a rectangular gate**.** (**D**) Click the **OK** button to obtain the FSC-A, FSC-W subset**.** (**E**) The FSC-A, FSC-W subset is obtained**.**

Supplementary Figure 14: Instructions for flow cytometry analysis software step number 4.2.3. (**A**) Double click the **FSC-A, FSC-W subset file**. (**B**) Click the **X-axis** and set it to "**SSC-A**"; click the **Y-axis** and set it to "**SSC-W**". (**C**) Click the **Create a rectangular gate** button to

create a rectangular gate. (**D**) Click the **OK** button to obtain the SSC-A, SSC-W subset. (**E**) The SSC-A, SSC-W subset is obtained.

Supplementary Figure 15: Instructions for flow cytometry analysis software step number 4.2.4. (**A**) Double click the **SSC-A, SSC-W subset file**. (**B**) Click the **X-axis** and set it to "**Hoechst Red-A**"; click the **Y-axis** and set it to "**Hoechst Blue-A**". (**C**) Click the **Create a polygon gate** button to create a polygon gate. (**D**) Click the **OK** button to obtain the Hoechst Red-A, Hoechst Blue-A subset. (**E**) The Hoechst Red-A, Hoechst Blue-A subset is obtained.

Supplementary Figure 16: Instructions for flow cytometry analysis software step number 4.2.5. (**A**) Click the **Open Layout Editor** button to open the layout editor. (**B**) Drag the **SSC-A, SSC-W subset sample file** to **Layout Editor**. (**C**) Click the **Click to save layout window to file** button to save the image results.

DISCUSSION:

There are several key points to keep in mind for the SP assay. The first is the selection of a proper blocker, such as Verapamil or Reserpine, for each cell line, because the "gate" location of the SP cells is determined according to the position at which SP cells disappear after the addition of the blocker. For the MDA-MB-231 cell line, Reserpine works well. However, for other cell lines, a different blocker might work better.

The second is the concentration of Hoechst 33342. The percentage of SP cells increased as the staining concentration of Hoechst 33342 decreased, as the representative data showed. This phenomenon can be explained by the dye absorption kinetics[24]. Changes in dye concentration and staining time affect enrichment of Hoechst 33342 in cells. Expelling of dye by SP cells through ABC transporters is an active energy-consuming transport process[24]. When the dye concentration is too high, the cells will be overstained, and more Hoechst 33342 needs to be pumped out. When ATP energy is exhausted, ABC transporters cannot pump the dye out of the cell continuously and the dye will accumulate in the cell, resulting in a lower proportion of SP cells until they finally disappear. When the concentration of Hoechst 33342 is too low, the cells are not completely stained, and the non-SP cells (which should be highly stained) appear in low-stained areas. Therefore, proper Hoechst 33342 staining concentration is closely related to the SP assay. Moreover, uptake and expulsion of Hoechst 33342 varies between cell types. Thus, proper concentration needs to be explored for different cell lines before the SP analysis.

The third is a good coefficient of variation (CV) of the flow cytometer, which is also critical for the SP analysis[25]. UV laser power is a strong criteria for better CVs[12]. This protocol uses a commercial flow cytometer (see **Table of Materials**) to perform the SP assay. In this cuvette-flow-cell instrument, we used the UV laser with a power of 15 mW to get the best CVs. In general, a relatively high UV laser power provides the optimal CVs. For example, 50–100 mW provides the optimal Hoechst signal on jet-in-air instruments[12]. Some lasers provide lower UV power, which can reduce CVs. For this reason, good laser alignment is critical.

The last is the influence of other factors during the experiment. Cell status, temperature, time of staining, operation of flow cytometry, and other factors may also affect the proportion and quality of the SP assay. For example, changes in cell viability during the preparation of cell suspensions will affect the ratio of SP cells. Therefore, the best experimental conditions need to be explored before performing SP analysis. Considering the above factors, the SP ratio of the same cell line measured by different laboratories may be different. For example the proportions of MDA-MB-231 cells and A549 cells were reported to be ~0.1%–4.8%[26–29] and ~0.8%–18%[30–34], respectively.

Researchers can modify this assay for different applications, such as the study of other tumor cell lines, or primary patient-derived tumor cells. If the protocol does not work well for the cells being tested, use MDA-MB-231 cells as the positive control and stain the cells following the given specifications. Because this protocol is very sensitive to the concentration of Hoechst 33342, staining temperature, and time check all these conditions closely. The percentage of SP in many types of human tumor cell lines is relatively low (~ 0%–37%)[19,26–36]. If an excessively high or low percentage of SP is observed, it may be due to inappropriate concentrations of Hoechst 33342 or blocker. If the problem seems to be related to the flow cytometer, obtain technical support.

Although the use of SP to analyze and separate CSCs is highly efficient, it still has certain limitations. The first is its high sensitivity to staining conditions[12]. The concentration of Hoechst 33342, cell status, temperature, time of staining, operation of flow cytometry, and the blocker selection affect the quality of SP analysis. The second is the cytotoxicity effect of Hoechst 33342 on cells. Hoechst 33342 is a DNA-binding dye, but is toxic to cells when it reaches high concentrations, thereby reducing cell activity[37].

In summary, SP analysis is one of the most commonly used methods in recent years to identify and purify CSCs in tumor cell lines. Although the method has some limitations, in the absence of specific CSCs surface markers, it is still a method for convenient, rapid, and cost-effective enrichment of CSCs. This method is beneficial for studying the biological functions of CSCs and for the identification of specific surface markers. Moreover, by detecting the effects of various signals on the SP ratio of tumor cells, it can provide clues to the regulatory effect of these signal pathways on CSCs features, and facilitate the discovery of new mechanisms, which can ultimately guide the targeted therapy of tumors.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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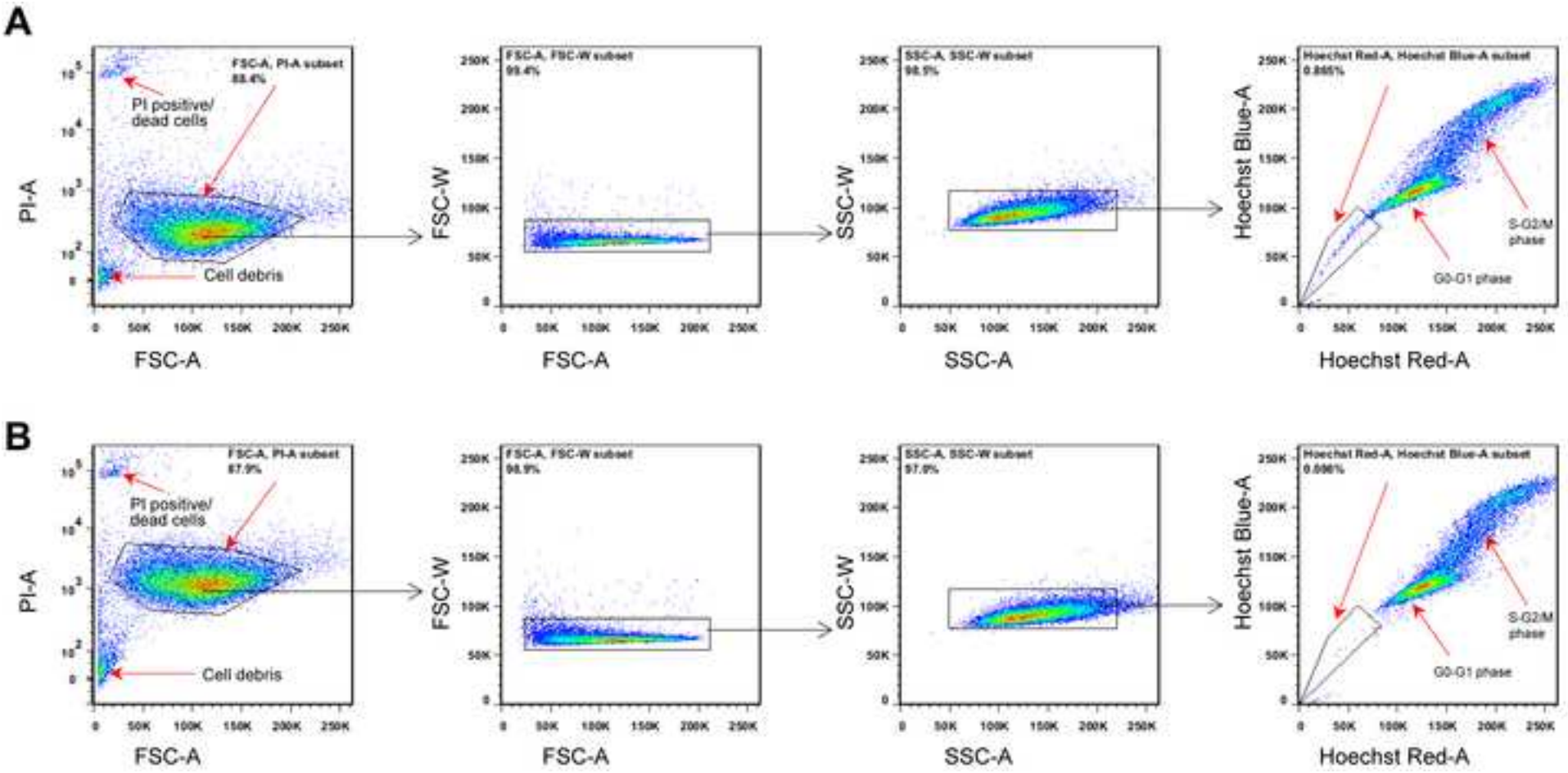
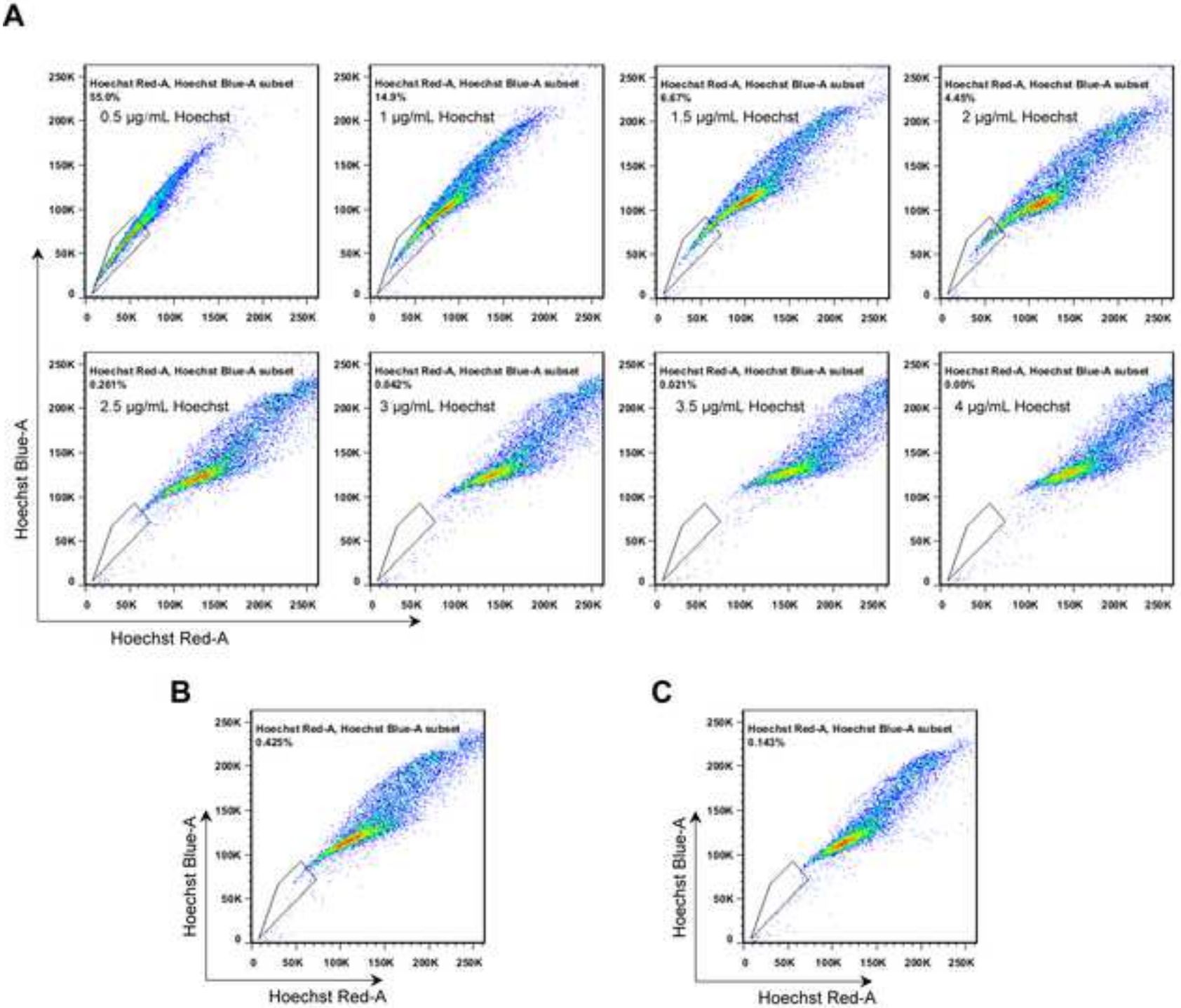


Figure 2



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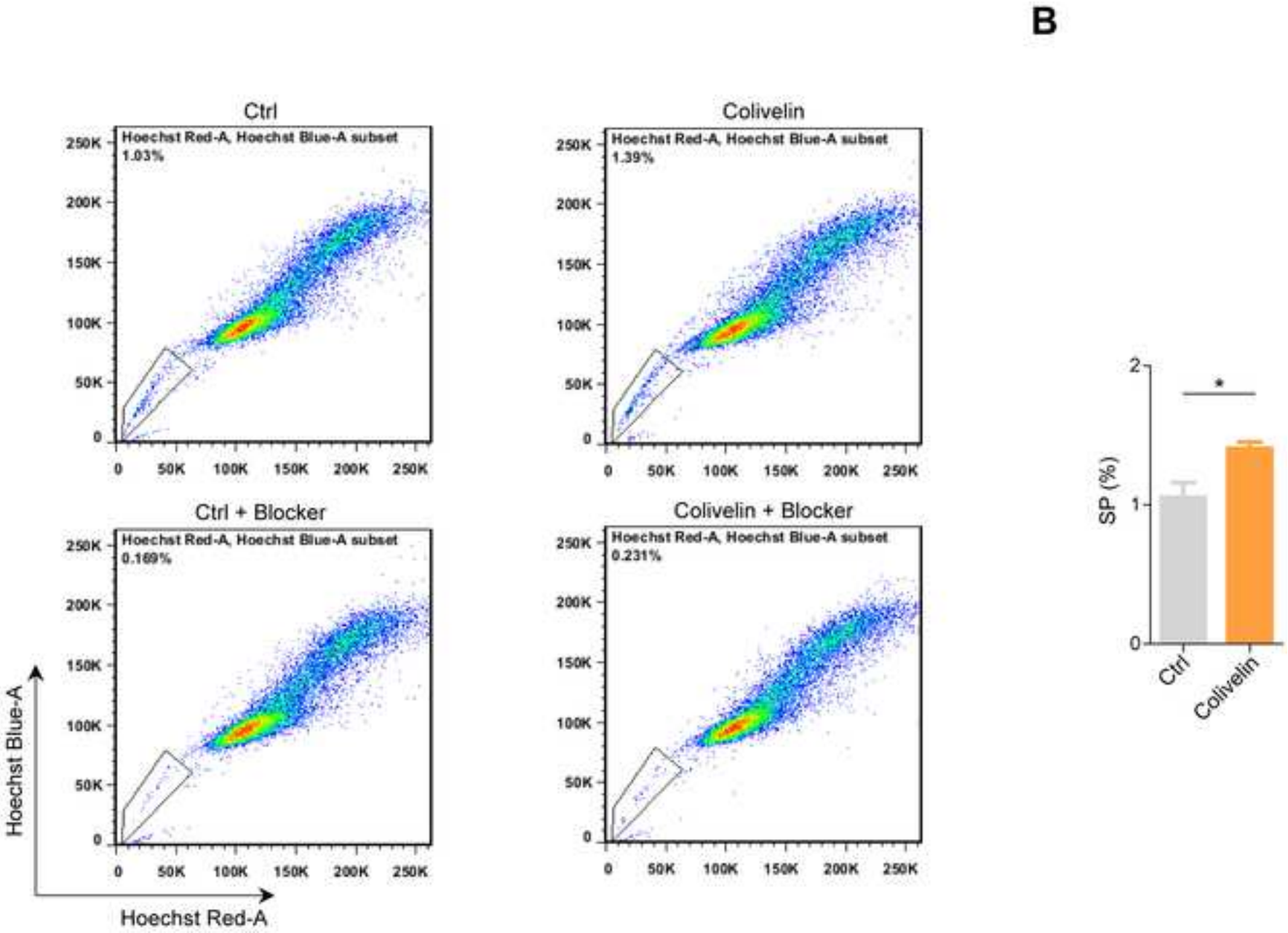

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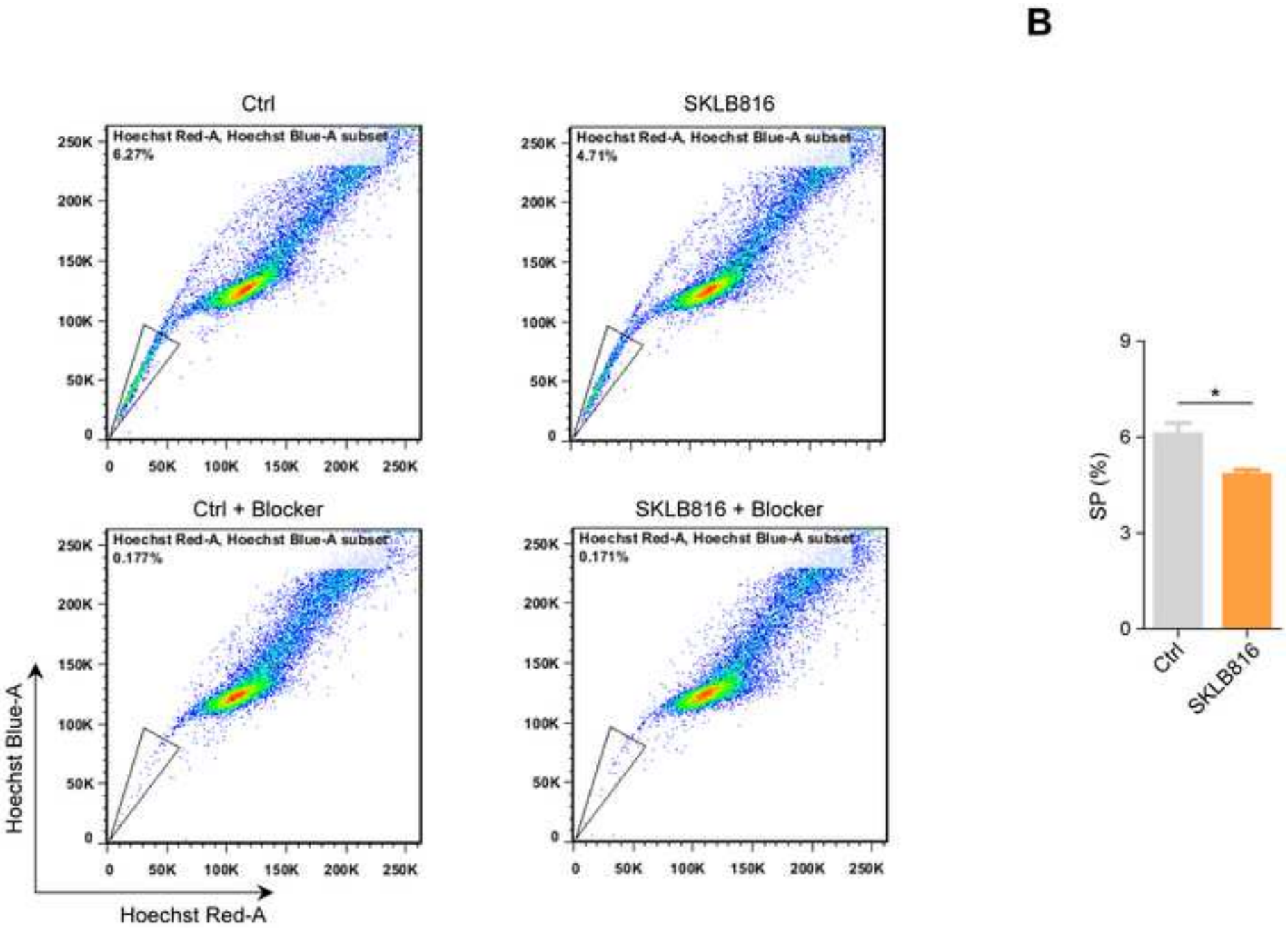

Name of Material/Equipment	Company	Name/Catalog Number
6 well cell culture plate	CORNING	3516
Colivelin	MCE	HY-P1061A
Fetal bovine serum (FBS)	(BIOIND)	04-001-1ACS
Flow cytometer	BD Biosciences	BD LSRFortessa
Flow cytometer software	BD Biosciences	FACSDiva
Flow cytometry analysis software	BD Biosciences	FlowJo
Hoechst33342	Sigma-Aldrich	B2261
Polystyrene round bottom test tube	CORNING	352054
Propidium iodide (PI)	Sigma-Aldrich	P4170
Reserpine	Sigma-Aldrich	83580
SKLB816	Provided by Dr. Shengyong Yang, Sichuan University	
Trypsin-EDTA (0.25%), phenol red	Gibco	25200072
Verapamil hydrochloride	Sigma-Aldrich	V4629

	Comments/Description
	9.5 cm^2 (approx.)
	Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala-Pro-Ala-Gly-Ala-Ser-Arg-Leu-Leu-Leu-Leu-Thr-Gly-Glu-Ile-Asp-Leu-Pro
	bisBenzimide H 33342 trihydrochloride 12 x 75 mm, 5mL 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (3β, 16β, 17α, 18β, 20α)-11,17-Dimethoxy-18-[(3,4,5-trimethoxybenzoyl)oxy]yohimban-16-carboxylic acid methyl ester
	5-[N-(3,4-Dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride

Editorial comments:

Dear Dr. Li,

Thank you for sending your figures. I think the manuscript doc and figure 1-4 are ok. However, supplementary figures look too crowded. I will request you to kindly have no more than 2-4 panels in one figure. In that case please modify the numbering in the manuscript.doc as well.

Thanks very much for your suggestion. We have separated the 2 supplementary figures into 16 figures with 1-4 panels in most Figures. In supplementary Figure 12-15, there are five panels (one big panel and four small panels) since they are from one substep and not easy to be separated. We have revised the numbering in the manuscript accordingly.

Regarding legend, it can go like: instructions for flow cytometer software step number 1.1.8- 1.1.9 (these can be directly derived from the protocol). Please place all the legends in the figure legend section.

Thanks for your suggestion, we have revised the figure legend accordingly.

Figure S1

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Supplementary Figure 1

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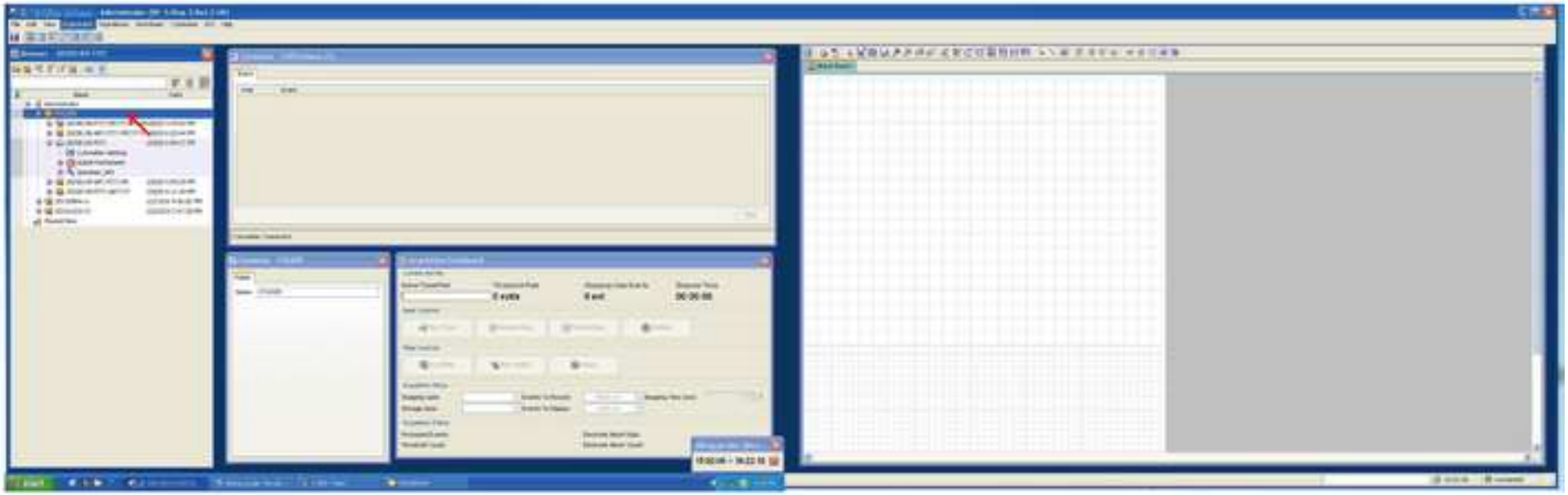

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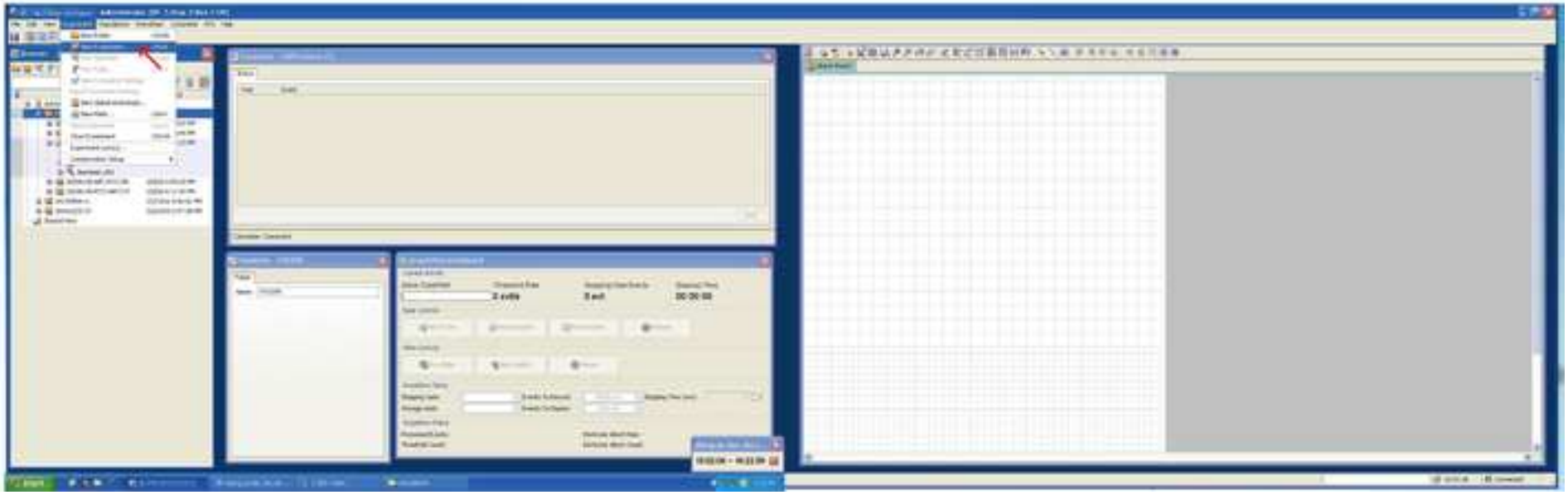

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Supplementary Figure 2

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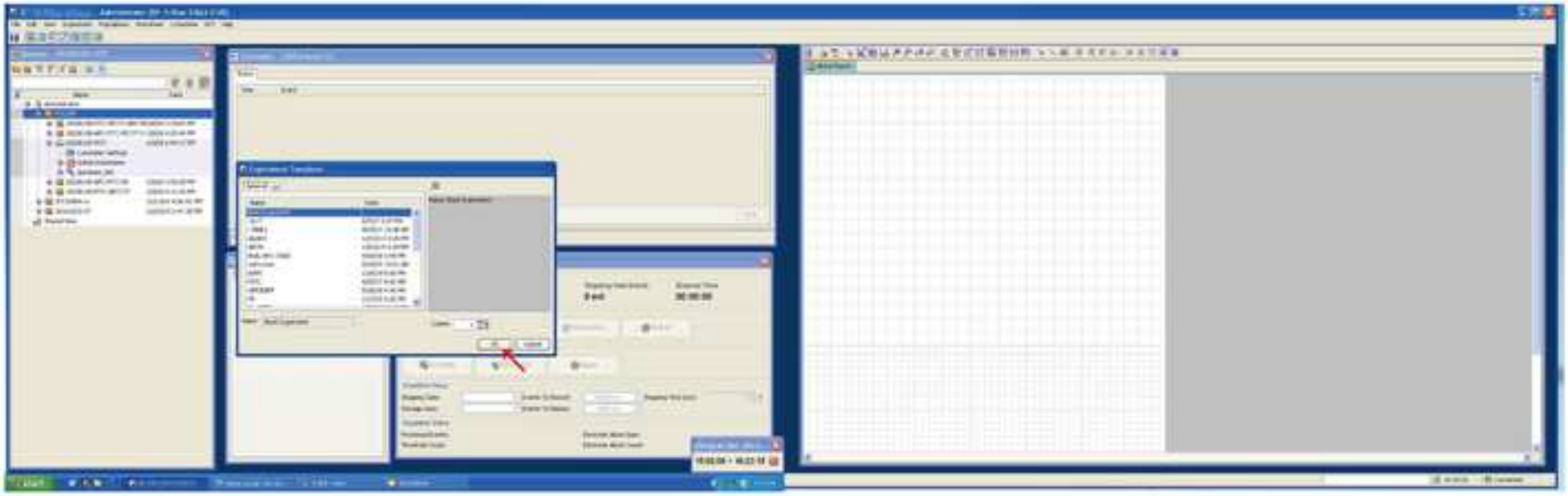

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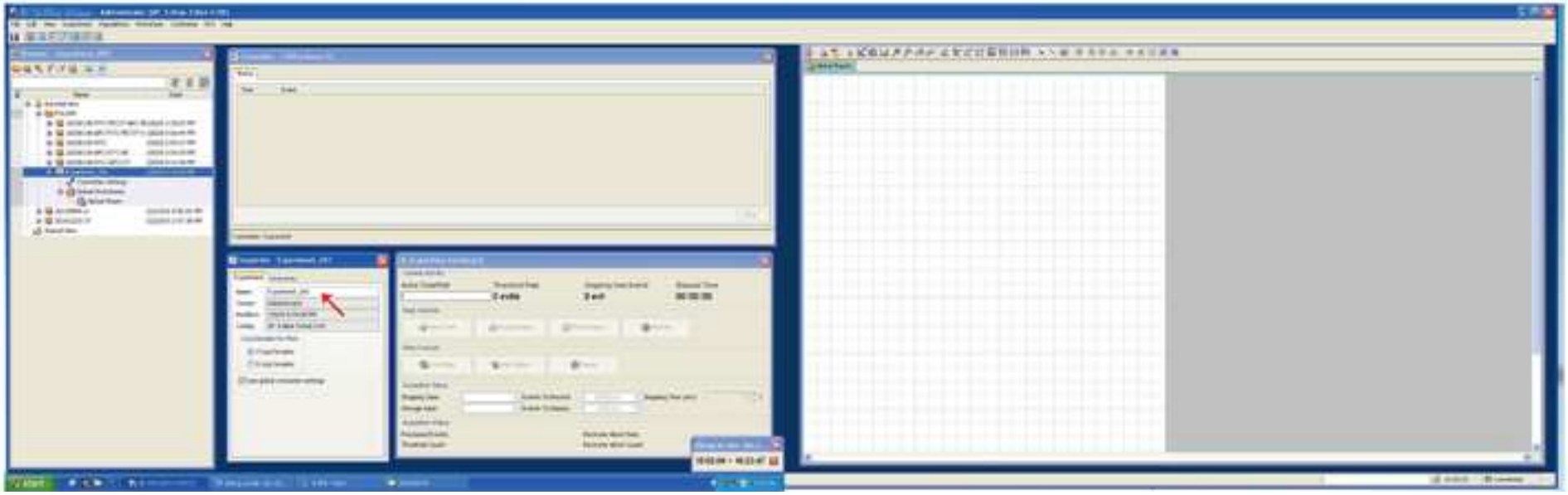

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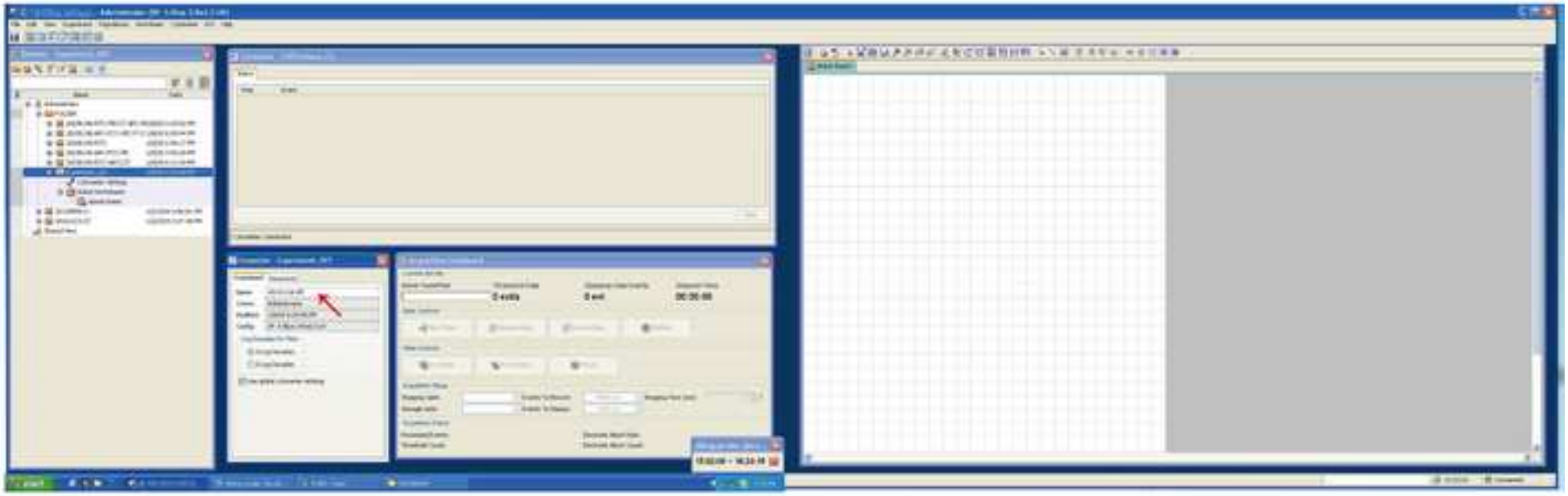

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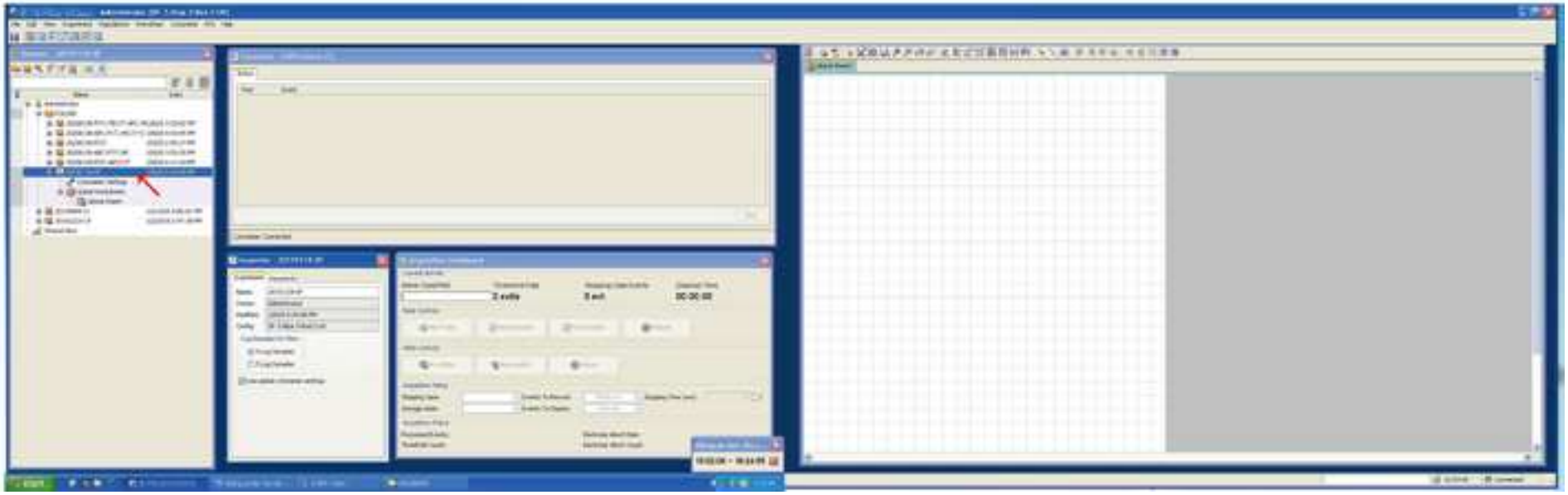

Figure S4

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Supplementary Figure 4

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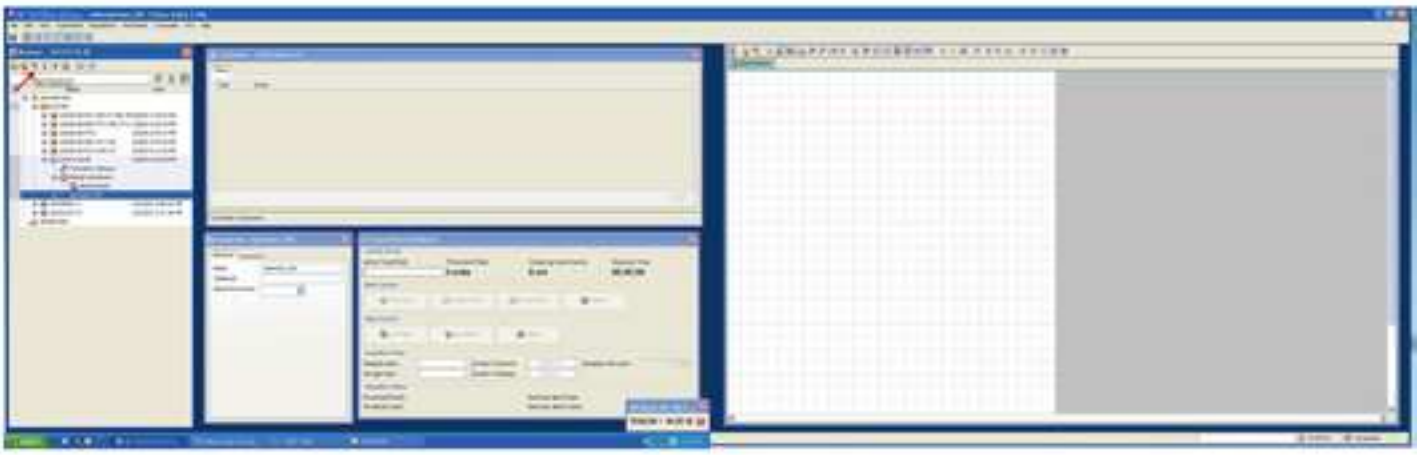

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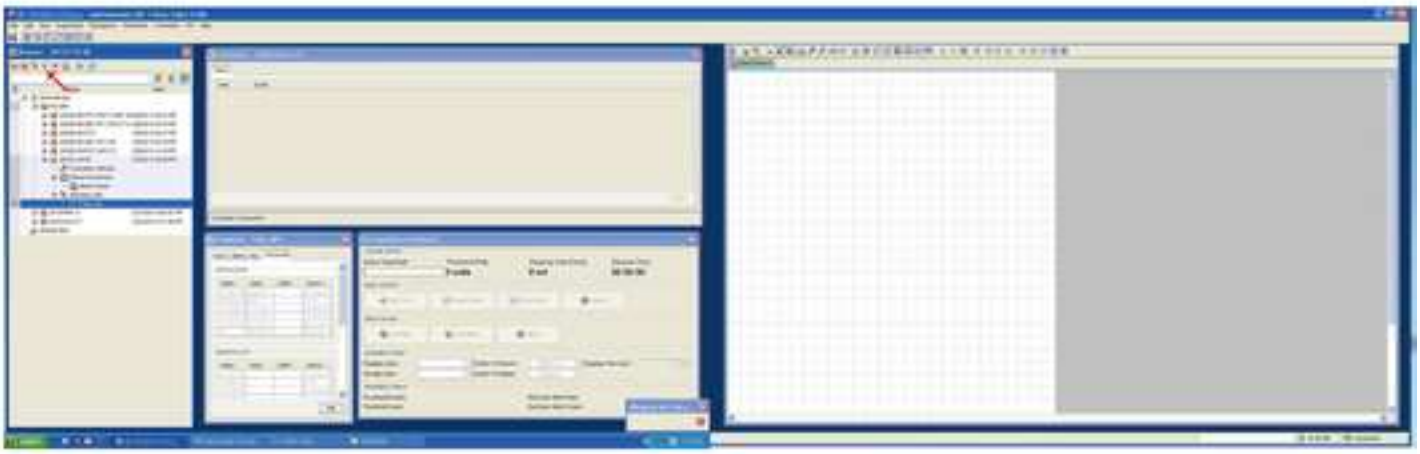

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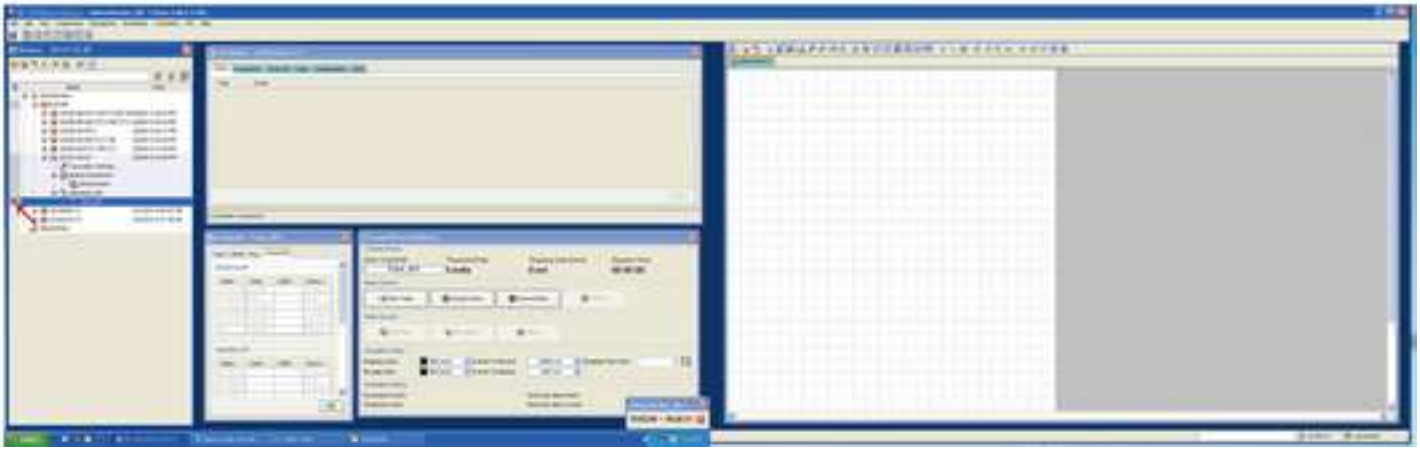

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Supplementary Figure 5

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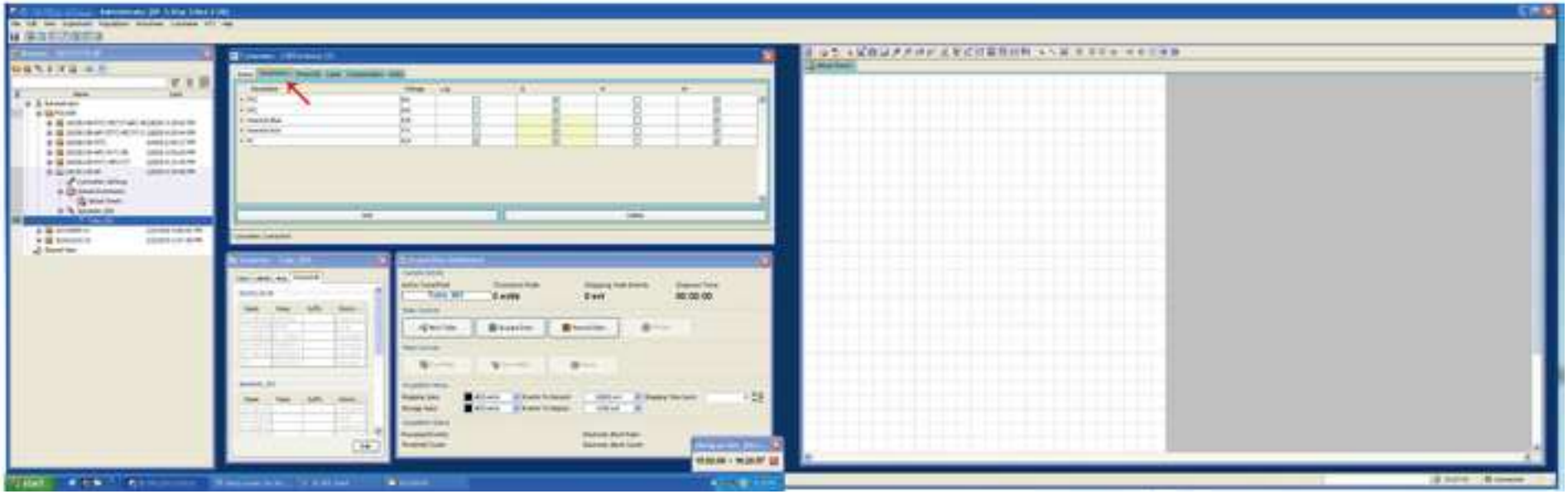

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Supplementary Figure 6

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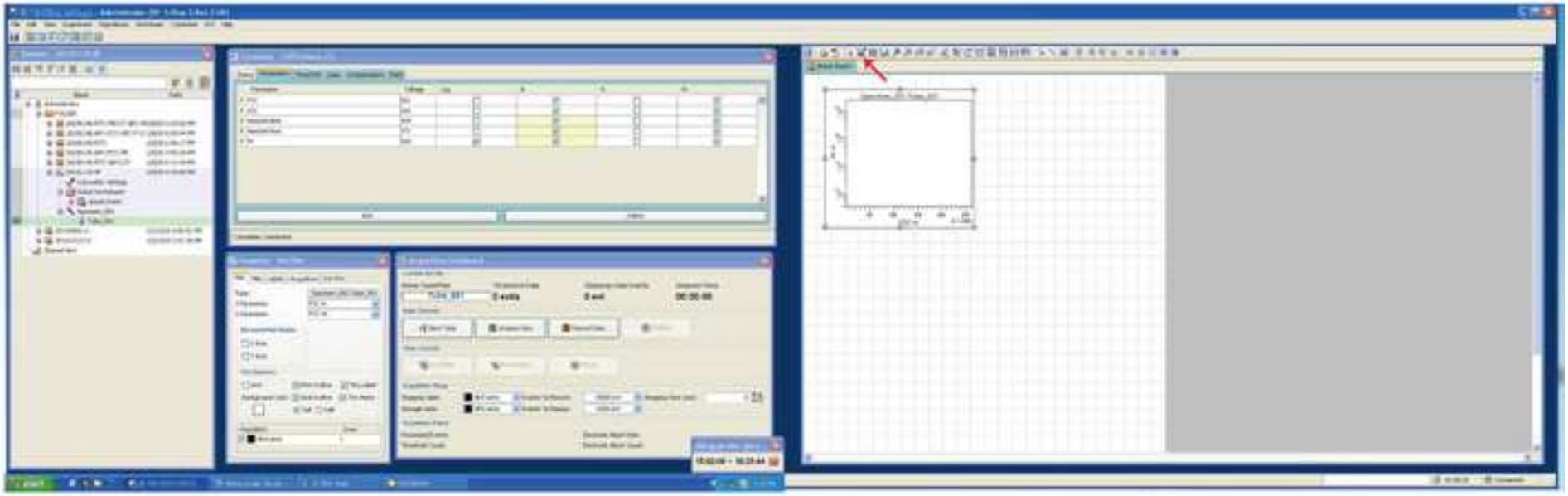

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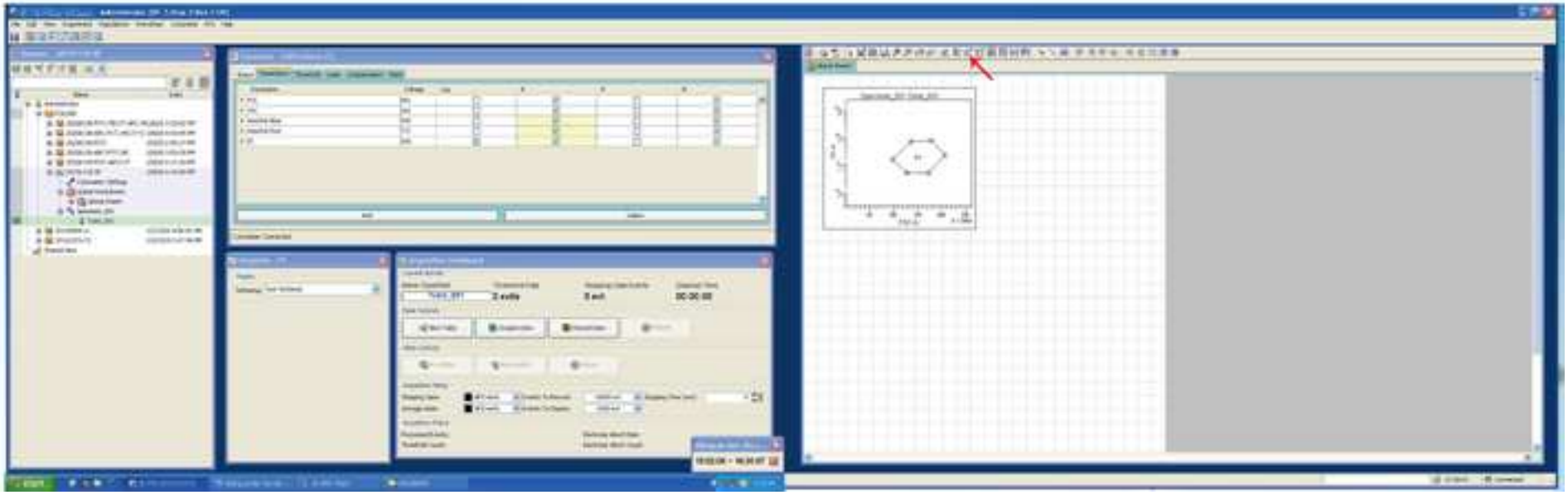

Figure S7

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Supplementary Figure 7

Figure S8

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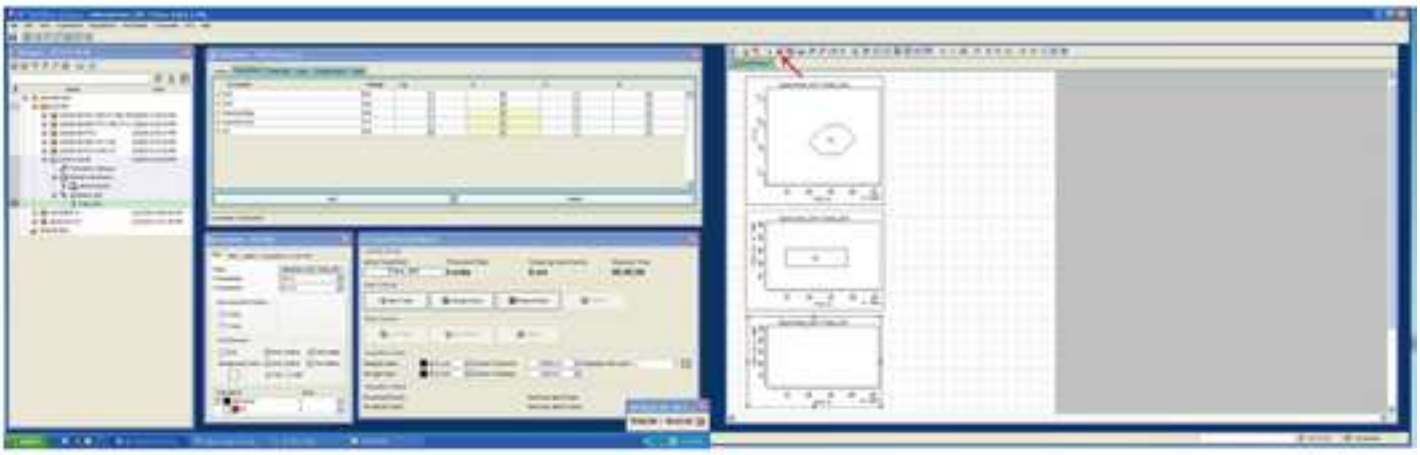
Supplementary Figure 8

A



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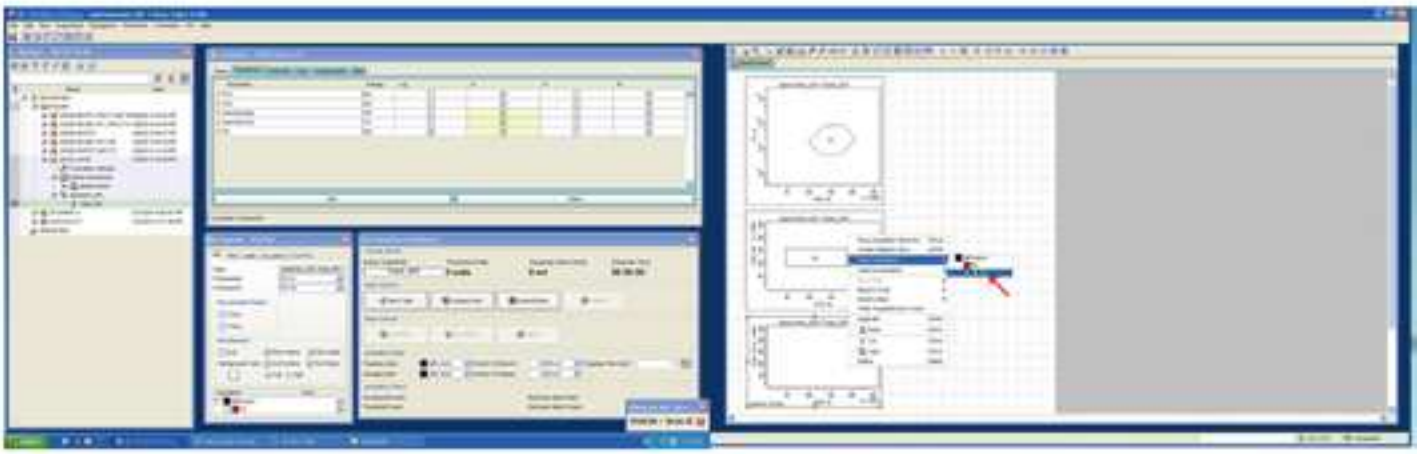

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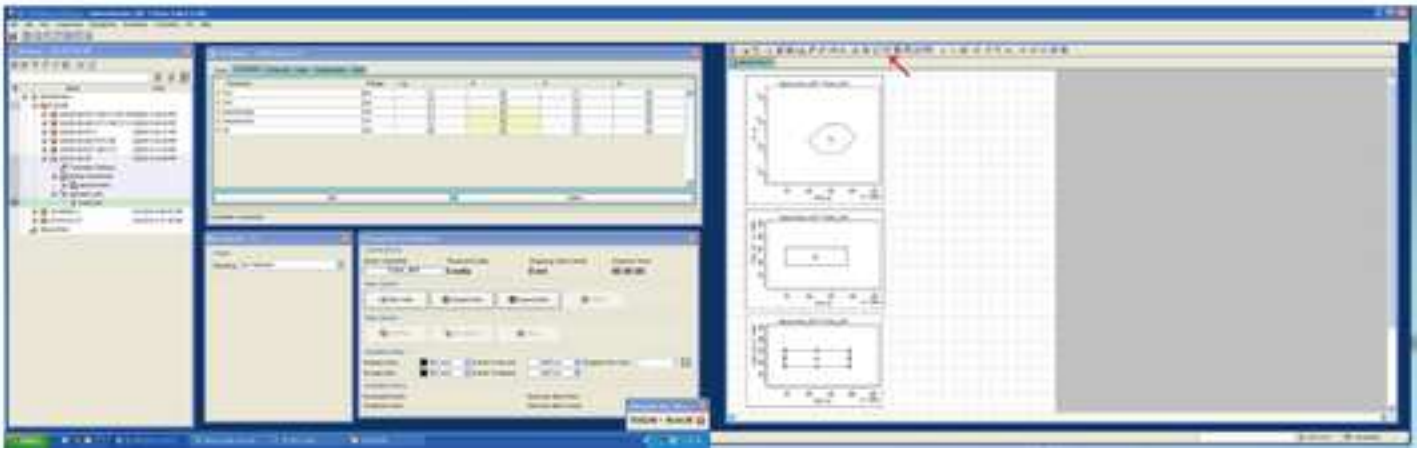

Figure S9

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Supplementary Figure 9

Figure S10

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Supplementary Figure 10

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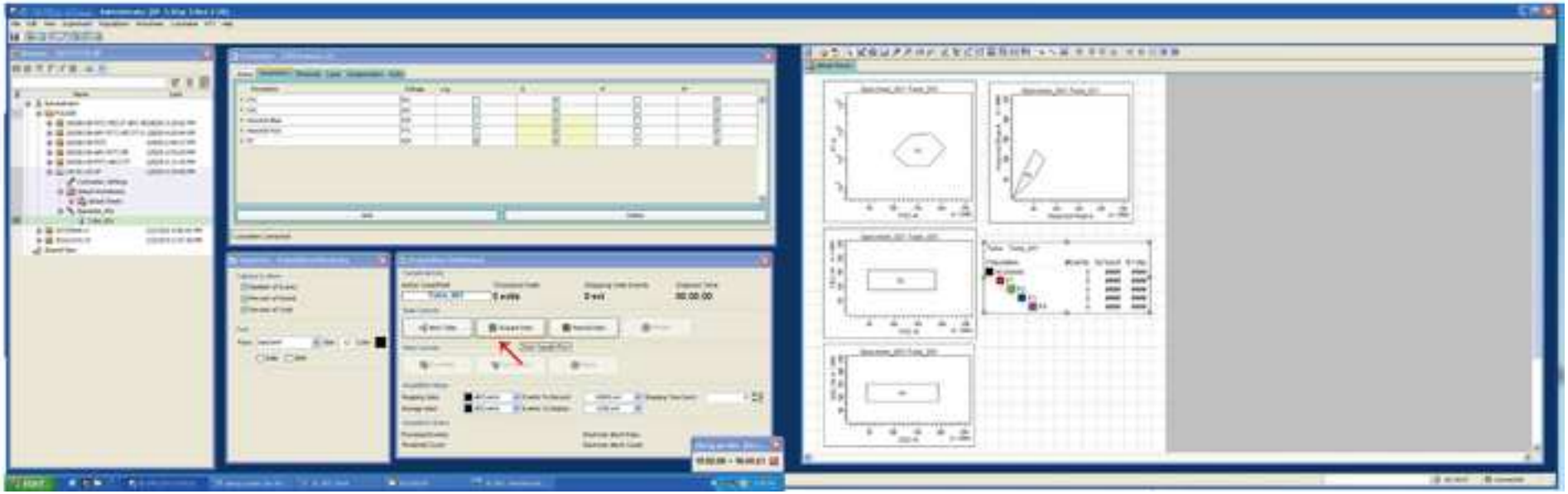

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Supplementary Figure 11

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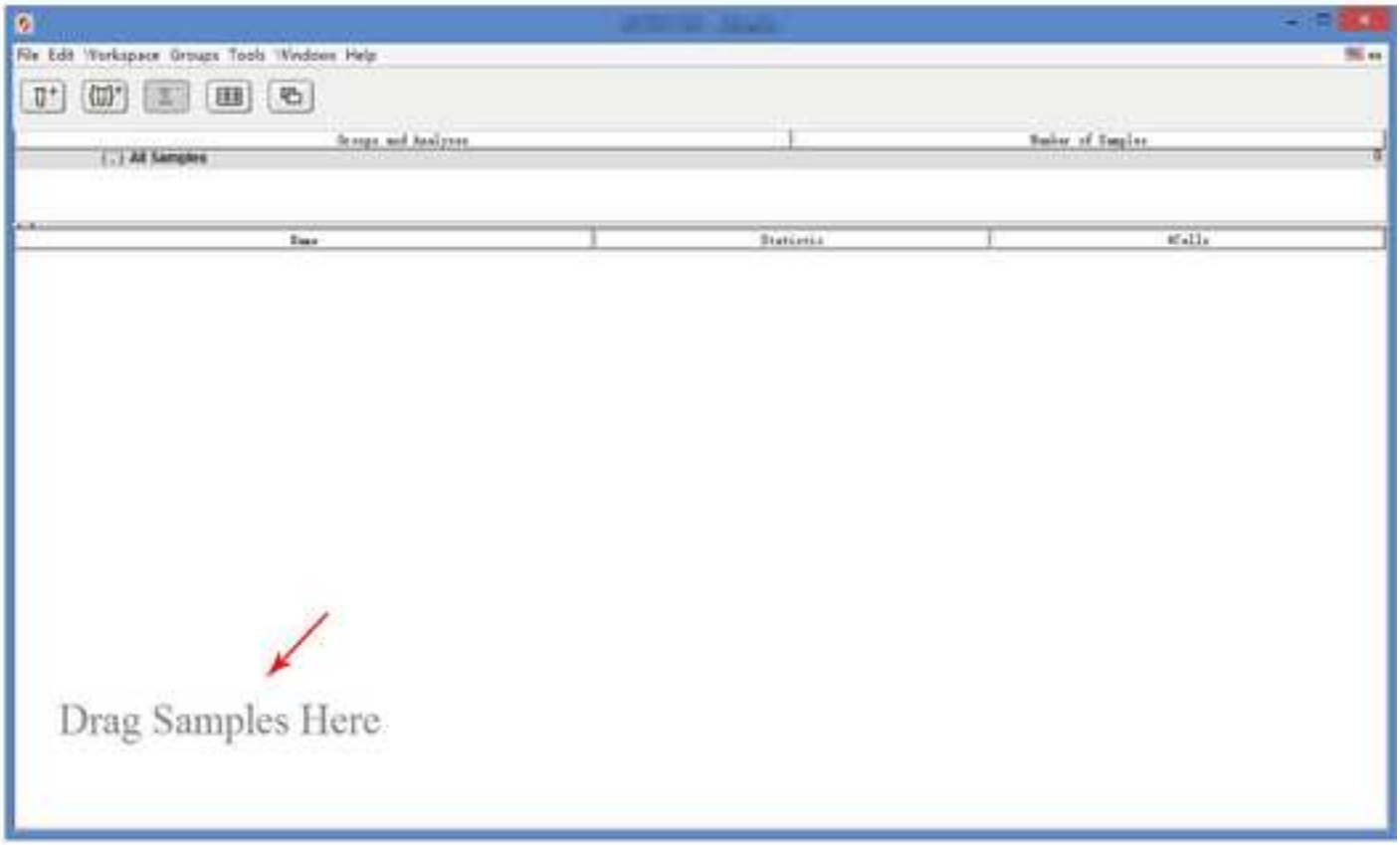

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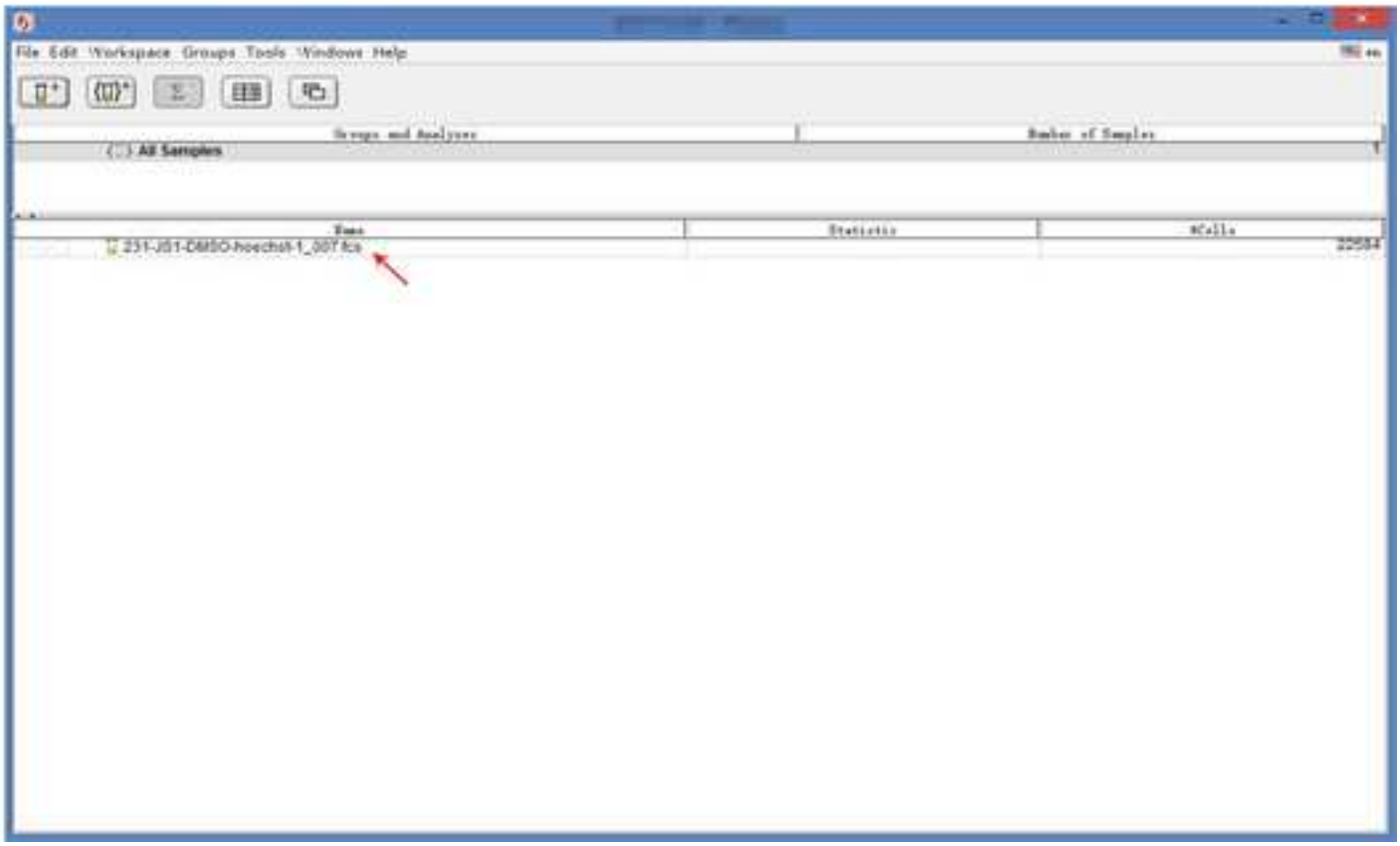

Figure S12

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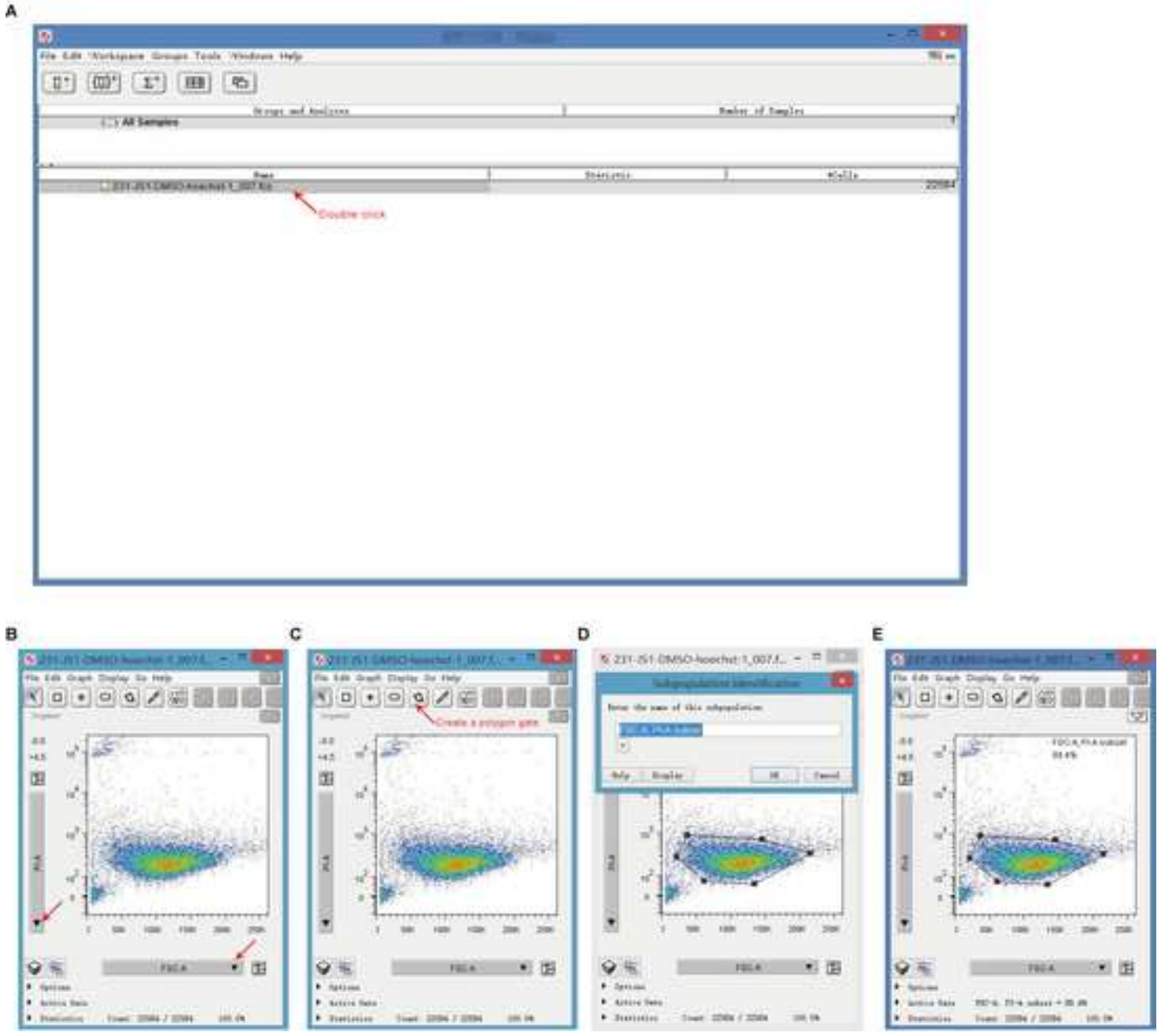
Supplementary Figure 12



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Supplementary Figure 13

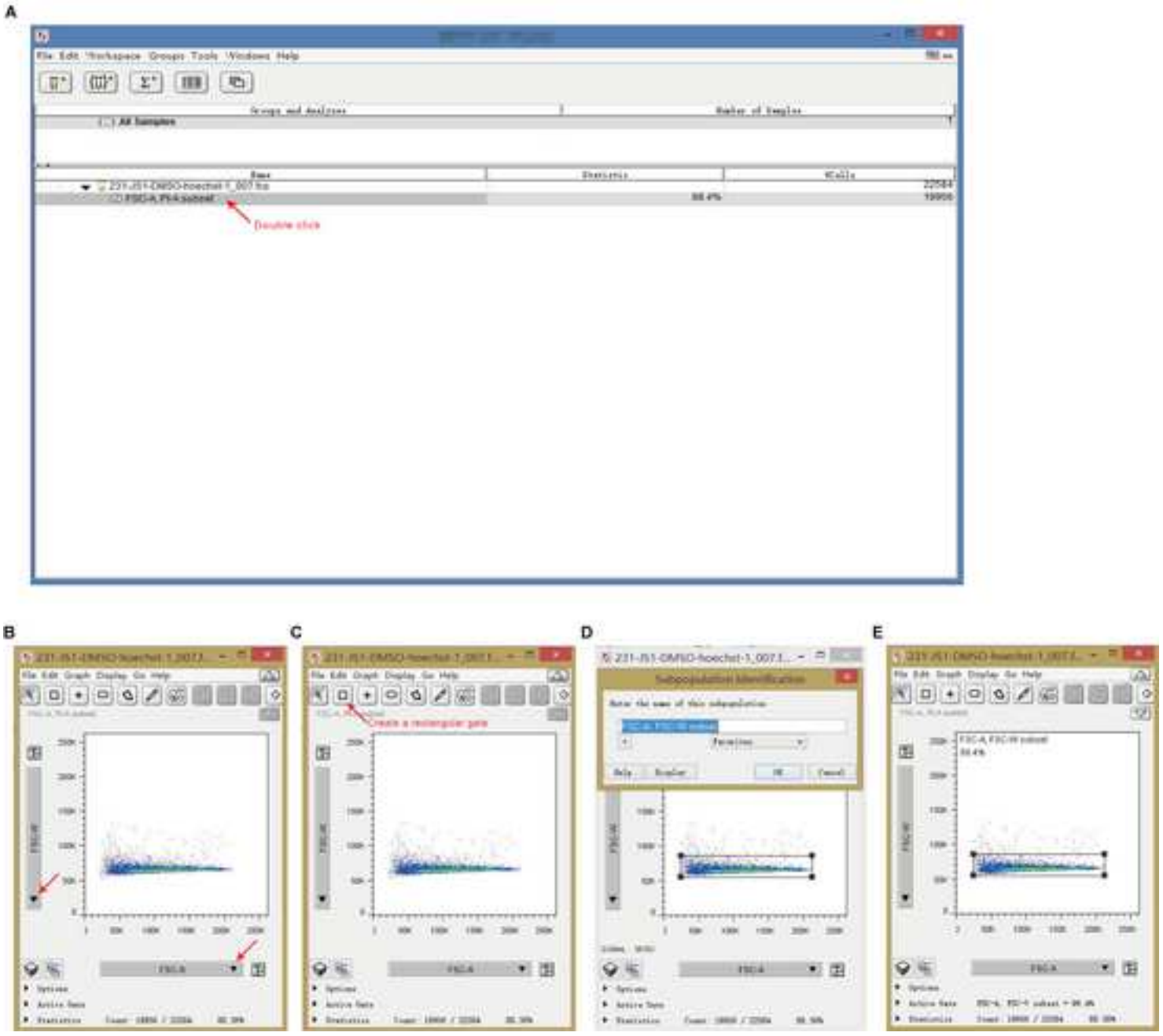

Figure S14

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Supplementary Figure 14

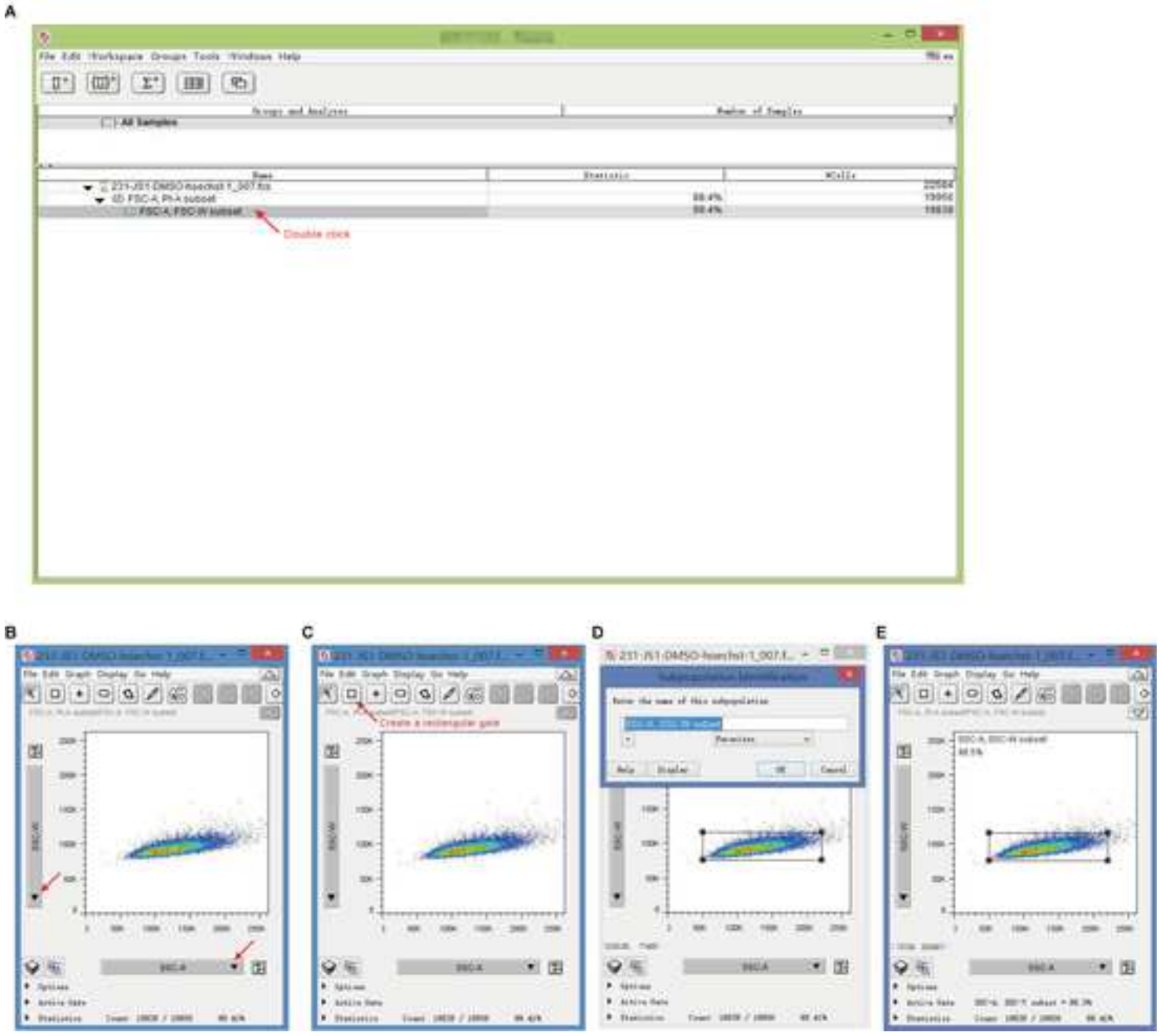

Figure S15

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Supplementary Figure 15

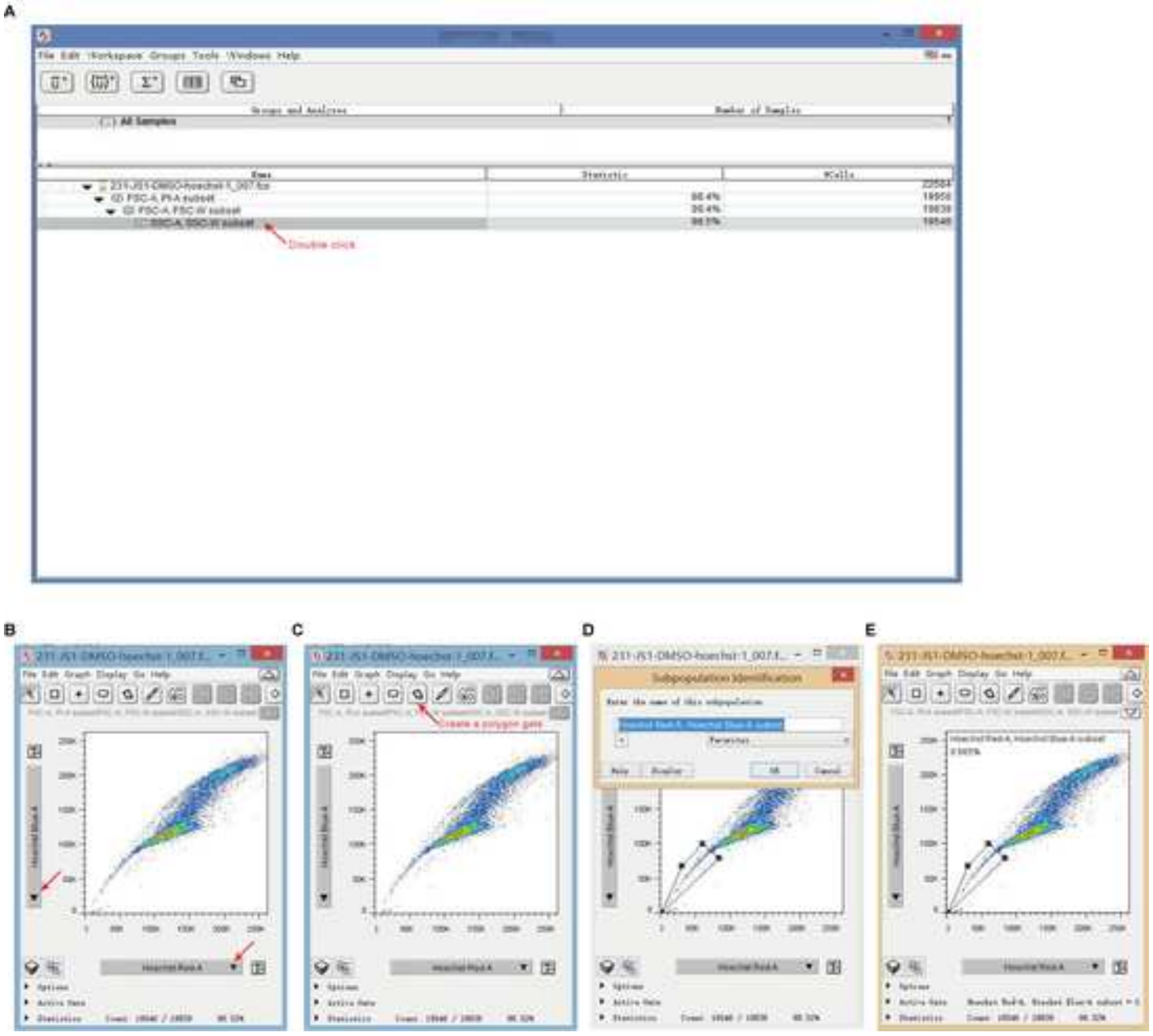

Figure S16

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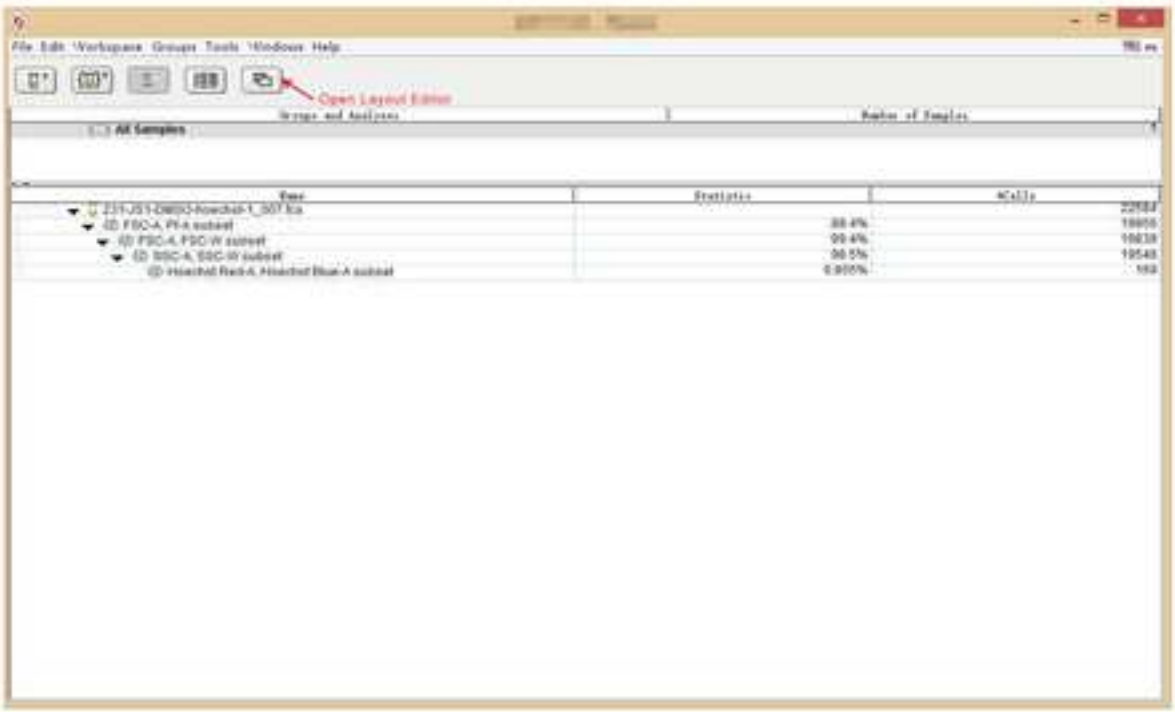
Supplementary Figure 16

A



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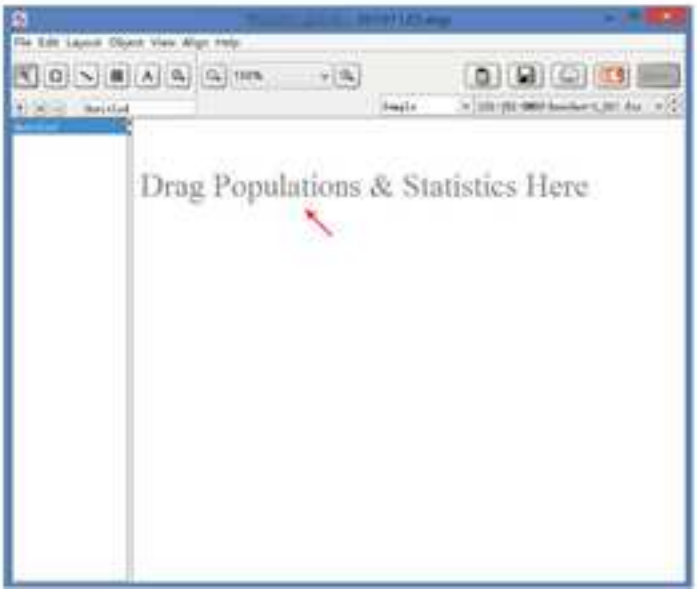
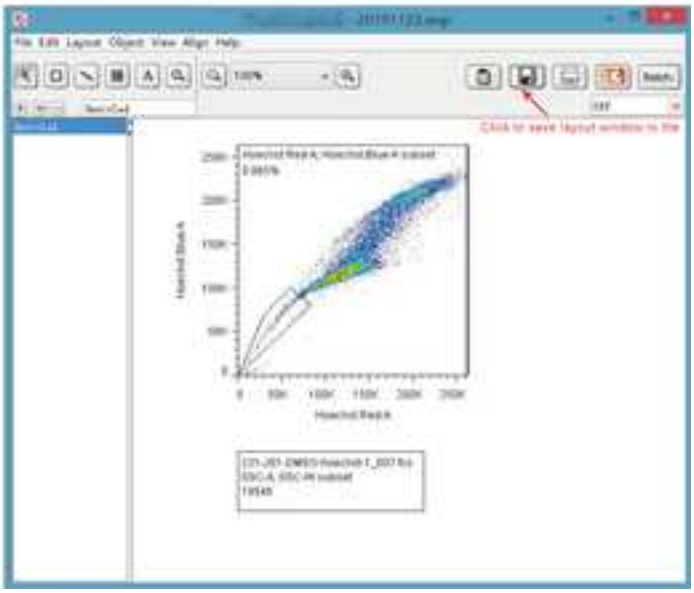


C



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CORRESPONDING AUTHOR

Name:	Na Li
Department:	Department of Pathophysiology
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