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Isolation of Murine Spermatogenic Cells Using a Violet-Excited Cell-Permeable DNA Binding Dye
--Manuscript Draft--

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Vineeta Bajaj, Ph.D.
Review Editor
JOVE

Dear Dr. Bajaj,

We would like to submit our revised manuscript titled "**Isolation of Murine Spermatogenic Cells Using a Violet-Excited Cell-Permeable DNA Binding Dye.**" for publication in *JOVE*.

We thank you for final instructions our manuscript (JoVE61666). This instruction enabled us to further improve the manuscript.

We provide, in full, responses and descriptions for how we address all of the Editorial comments.

We also addressed all the comments in the text. Please see the comments in the text. We highlighted changes in red.

We thank you again for your immensely helpful criticism, and we thank you for your consideration.

Most sincerely,

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1 **TITLE:**
2 Isolation of Murine Spermatogenic Cells Using a Violet-Excited Cell-Permeable DNA Binding Dye
3

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35 **KEYWORDS:**
36 spermatogenesis, meiosis, spermatocytes, spermatids, fluorescence-activated cell sorting, FACS,
37 DyeCycle Violet, DCV
38

39 **SUMMARY:**
40 Here we present a simple and efficient method to isolate live meiotic and post-meiotic germ cells
41 from adult mouse testes. Using a low-cytotoxicity, violet-excited DNA binding dye and
42 fluorescence-activated cell sorting, one can isolate highly enriched spermatogenic cell
43 populations for many downstream applications.
44

ABSTRACT:

Isolation of meiotic spermatocytes is essential to investigate molecular mechanisms underlying meiosis and spermatogenesis. Although there are established cell isolation protocols using Hoechst 33342 staining in combination with fluorescence-activated cell sorting, it requires cell sorters equipped with an ultraviolet laser. Here we describe a cell isolation protocol using the DyeCycle Violet (DCV) stain, a low cytotoxicity DNA binding dye structurally similar to Hoechst 33342. DCV can be excited by both ultraviolet and violet lasers, which improves the flexibility of equipment choice, including a cell sorter not equipped with an ultraviolet laser. Using this protocol, one can isolate three live-cell subpopulations in meiotic prophase I, including leptotene/zygotene, pachytene, and diplotene spermatocytes, as well as post-meiotic round spermatids. We also describe a protocol to prepare single-cell suspension from mouse testes. Overall, the procedure requires a short time to complete (4-5 hours depending on the number of needed cells), which facilitates many downstream applications.

INTRODUCTION:

Spermatogenesis is a complex process wherein a small population of spermatogonial stem cells sustain continuous production of a large number of sperm throughout adult life[1,2]. During spermatogenesis, dynamic chromatin remodeling takes place when spermatogenic cells undergo meiosis to produce haploid spermatids[3-5]. Isolation of meiotic spermatocytes is essential for molecular investigation, and several different approaches to isolate meiotic spermatocytes have been established, including sedimentation-based separation[6,7] and fluorescence-activated cell sorting (FACS)[8-17]. However, these methods have technical limitations. While sedimentation-based separation yields a large number of cells[5-7], it is labor intensive. The established FACS-based method uses Hoechst 33342 (Ho342) to separate meiotic spermatocytes based on DNA content and light scattering properties and requires FACS cell sorters equipped with an ultraviolet (UV) laser[8-11]. Alternative FACS-based methods require transgenic mouse lines that express florescent proteins, synchronization of spermatogenesis[12], or cell fixation and antibody labeling that is not compatible with isolation of live cells[13]. While there is another alternative method using a cell-permeable DNA binding dye, DyeCycle Green stain[14-17], this method is recommended for the isolation of spermatogenic cells from juvenile testis. Therefore, there is a critical need to develop a simple and robust isolation method for live meiotic spermatocytes that can be applied to any mouse strain of any age and that can be performed using any FACS cell sorter.

Here we describe such a long-sought cell isolation protocol using the DyeCycle Violet (DCV) stain. DCV is a low cytotoxicity, cell-permeable DNA binding dye structurally similar to Ho342 but with an excitation spectrum shifted toward the violet range[18]. In addition, DCV has a broader emission spectrum compared to DCG. Thus, it can be excited by both UV and violet lasers, which improves the flexibility of equipment, allowing the use of an FACS cell sorter not equipped with a UV laser. The DCV protocol presented here uses two-dimensional separation with DCV blue and DCV red, mimicking the advantage of the Ho342 protocol. With this advantage, our DCV protocol allows us to isolate highly enriched germ cells from the adult testis. We provide a detailed gating protocol to isolate live spermatogenic cells from adult mouse testes of one mouse (from two testes). We also describe an efficient and quick protocol to prepare single-cell suspension from mouse testes that can be used for this cell isolation. The procedure requires a short time to

89 complete (preparation of single cell suspension - 1 hour, dye staining - 30 min, and cell sorting -
90 2-3 hours: total - 4-5 hours depending on the number of needed cells; **Figure 1**). Following cell
91 isolation, a wide range of downstream applications including RNA-seq, ATAC-seq, ChIP-seq, and
92 cell culture can be completed.
93
94 **PROTOCOL:**
95 This protocol follows the guidelines of the Institutional Animal Care and Use Committee (protocol
96 no. IACUC2018-0040) at Cincinnati Children's Hospital Medical Center.
97
98 **1. Equipment and reagent setup for the preparation of testicular cell suspension**
99
100 1.1. Prepare each enzyme stock in 1x Hanks' Balanced Salt Solution (HBSS) and store at -20 °C.
101 (**Table1**).
102
103 NOTE: Prepare any time before the experiment.
104
105 1.2. One day before the experiment: Coat collecting tubes (1.5 mL tubes) with Fetal bovine
106 serum (FBS) at 4 °C overnight.
107
108 1.3. On the day of the experiment: Set the water bath to 37 °C.
109
110 1.4. Pre-warm Dulbecco's Modified Eagle Medium (DMEM) at 37 °C and prepare 2 mL of 1x
111 dissociation buffer for each sample right before use (**Table 1**) in 15 mL centrifuge tube.
112
113 NOTE: 2 mL dissociation buffer is prepared for two testes. For the dissociation buffer recipe,
114 please refer to **Table 1**.
115
116 **2. Animal dissection and preparation of testicular cell suspension**
117
118 2.1. Sacrifice an 8-week-old male mouse by leaving in a carbon dioxide chamber for at least 10
119 min.
120
121 2.2. Remove both testes and place on a 60 mm Petri dish containing 2 mL of ice-cold phosphate-
122 buffered saline (PBS).
123
124 2.3. Remove the tunica albuginea from the testes. Slightly disperse the seminiferous tubules by
125 gently separating with forceps.
126
127 2.4. Transfer the seminiferous tubules to a 100 mm Petri dish with a new drop of ice-cold PBS
128 and untangle seminiferous tubules gently with forceps. Repeat this wash 3 times to remove
129 interstitial cells as much as possible.
130
131 2.5. Incubate untangled seminiferous tubules in a 15 mL tube containing 2 mL of dissociation
132 buffer at 37 °C for 20 min.

2.6. Gently pipette the tubules 20 times using a 1000 µL micropipette.

2.7. Incubate for 6 min. Repeat gentle pipetting 20 times.

2.8. Incubate for 3 min. Repeat gentle pipetting 10 times until no visible chunks remain.

2.9. Add 10 mL of FACS buffer to the suspension. Centrifuge at 300 x g for 5 min at room temperature and discard the supernatant.

2.10. Repeat step 2.9 twice to remove the spermatozoa and as much debris as possible.

3. Cell staining

3.1. Resuspend the cell pellet with 3 mL of FACS buffer (**Table 1**) and filter the cell suspension through a 70 µm nylon cell strainer into a 50 mL tube.

NOTE: The expected cell yield is approximately 100 million cells per two testicles (from an 8-week-old B6 wild-type mouse).

3.2. Count the cell number and split 10% of the cells into a new tube as the unstained negative control and leave on ice.

3.3. Add 6 µL of DCV stain (the original concentration is 5 mM) to the remaining cell suspension and mix well. The final concentration is 10 µM, and the capacity is approximately 100 million cells.

3.4. Incubate at 37 °C for 30 min in the dark. Gently shake the cell suspension every 10 min.

3.5. After incubation, without a wash step, directly add 5 µL of DNase I (the stock concentration is 10 mg/mL) to the cell suspension and filter the cells into a 5 mL FACS tube through the 35 µm nylon mesh cap. Keep the samples on ice until sorting.

4. Flow cytometry and experimental gates

4.1. Prepare the FACS cell sorter. Ensure that FACS cell sorter is equipped with Excitation optics: Violet (405-nm) laser; Detection optics: filter combination of 450/50 bandpass [same filter set of 4',6-diamidino-2-phenylindole (DAPI) for DCV-blue detection] and 665/30 bandpass [same filter set of Allophycocyanin (APC) for DCV-red detection]. Here we use Sony SH800S cell sorter as an example.

4.2. Create a new experiment and set up the following working plots displaying parameters to be optimized: Click "**New Density**" on the "**Worksheet Tools**" menu bar to create a forward scatter - area (FSC-A) vs. back scatter – area (BSC-A) density plot on a linear scale. Click "**New Histogram**" to create a DCV-blue histogram plot on logarithmic scale.

177

178   4.3. Briefly vortex the unstained negative control (from step 3.2) and load the sample.

179

180   4.4. Click "**Start**" and "**Record**" to begin processing the unstained sample. While the sample is
181   running, click "**Detector & Threshold Settings**" to optimize FSC and BSC voltages by adjusting
182   both the photomultiplier tube (PMT) voltages up or down to place the unstained cells on the
183   scale of the FSC/BSC plot.

184

185   4.5. Adjust the PMT voltage up and down on "**FL1: DAPI**" while the sample is running to locate
186   the position of the DCV-negative population in the first decade of the DCV-blue histogram
187   logarithmic plot (**Figure 2A**). After completing the PMT voltage adjustment, click "**Stop**" to unload
188   the unstained sample.

189

190   4.6. Briefly vortex the sample (from step 3.6) and load the sample.

191

192   4.7. Click "**Next Tube**" to create a new worksheet for the DCV-stained sample; and click "**Start**"
193   and "**Record**" to acquire the DCV-stained sample, record ≥ 1 x 10^6 events. Add the following
194   working plots: Click "**New Density**" on the "**Worksheet Tools**" menu bar to create an FSC-H
195   (height) vs. FSC-W (width) density plot on a linear scale; Click "**New Density**" to create a DCV-
196   blue vs. DCV-red density plot on a linear scale. After recording ≥ 1 x 10^6 events, click "**Stop**" and
197   unload the sample.

198

199   4.8. On the FSC-A vs. BSC-A density plot, click "**Polygon**" on the "**Plot Tools**" menu bar to draw a
200   big gate "**Cells**" to include most cells and exclude small debris (**Figure 2B**). Apply the gate "**Cells**"
201   to FSC-H vs. FSC-W density plot. Click "**Rectangle**" to draw a "**Single Cells**" gate to exclude non-
202   single cells (**Figure 2C**).

203

204   4.9. Apply "**Single Cells**" gate to DCV-blue vs. DCV-red density plot and adjust the scale to capture
205   an extended profile as shown in **Figure 2D**. Click "**Polygon**" on the "**Plot Tools**" menu bar to draw
206   a "**DCV**" gate to exclude the unstained cells and side population **(Figure 2D).**

207

208   4.10. Apply "**DCV**" gate to DCV-blue histogram plot on a linear scale. The three major peaks refer
209   to different DNA content: 1C, 2C, and 4C (**Figure 2E**).

210

211   4.11. Click "**New Density**" to create a DCV-blue vs. DCV-red density plot on a linear scale and
212   apply "**DCV**" gate to perform back gating from **Figure 2E** to locate the 1C and 4C populations
213   (**Figure 2F**). Click "**Ellipse**" to draw a gate on the 1C population, which is within a condensed area
214   (gate 1C). Click "**Polygon**" to draw a gate on the 4C population which is a continuous curve (gate
215   4C).

216

217   4.12. Click "**New Density**" to create a DCV-blue vs. DCV-red density plot on a linear scale and
218   apply "4C" gate; adjust the scale to zoom in and click "**Polygon**" to draw a "4C_1" gate for more
219   precise selection (**Figure 2G**).

220

221 <mark>4.13. Click "**New Density**" to create a new FSC-A vs. BSC-A density plot on a linear scale and apply</mark>
222 <mark>"4C_1" gate; there will be three enriched populations separated by size corresponding to</mark>
223 <mark>leptotene (L) /zygotene (Z), pachytene (P) and diplotene (D) spermatocytes. Click "**Polygon**" or</mark>
224 <mark>"**Ellipse**" to draw 3 gates: "L/Z", "P", and "D" based on the growing size (**Figure 2H**).</mark>
225
226 <mark>4.14. Click "**New Dot Plot**" to create a DCV-blue vs. DCV-red color dot plot on a linear scale to</mark>
227 <mark>apply gate and ensure the three populations are in a continuous order within the "4C_1" gate</mark>
228 <mark>(**Figure 2I**).</mark>
229
230 <mark>4.15. Similarly, for the 1C population, click "**New Density**" to create a new FSC-A vs. BSC-A density</mark>
231 <mark>plot on a linear scale and apply "1C" gate, select the unified size of cells as pure round spermatid</mark>
232 <mark>population, and click "**Ellipse**" to draw an "**RS**" gate (**Figure 2J**).</mark>
233
234 **5. Sort male germ cell subpopulations**
235
236 5.1. Prepare 1.5 mL tubes containing 500 µL 50% FBS for cell collection and load the collection
237 tube into the collector and click "**Load Collection**".
238
239 5.2. Click "**Next Tube**", "**Start**", "**Record**" and "**Sort Start**". For using a two-way system that allows
240 two populations of interest to be sorted at the same time into the collection device, follow
241 pairwise combinations: leptotene (L) /zygotene (Z) and pachytene (P) spermatocytes based on
242 L/Z and P back-gates; Round spermatids (RS) and diplotene (D) spermatocytes based on RS and
243 D back-gates.
244
245 5.3. While the sample is running, adjust the flow rate to ~3000 events/s to get the most efficient
246 sorting.
247
248 **6. Purity analysis of sorted cells**
249
250 6.1. Collect ≥ 10,000 cells/each population. Perform cell immunostaining to confirm the substage.
251
252 6.2. Centrifuge at 300 x g for 5 min at 4 ˚C and carefully discard the supernatant, keeping around
253 110 µL of the liquid in the bottom of the tube.
254
255 6.3. Take a 10 µL drop of cell suspension to observe under microscope and evaluate the overview
256 cell morphology and number.
257
258 6.4. Apply 100 µL of cell suspension to each of the sample chamber slides (see **Table of Materials**).
259
260 6.5. Spin the samples at 30 x g for 5 min at room temperature. Draw a circle around the cell with
261 a hydrophobic pen. Dry slides on lab bench for a few minutes at room temperature.
262
263 6.6. Drop 50 µL of PBS in the circle of the slide and tap off.
264

265 6.7. Add primary antibody solution (Dilute primary antibodies with 5% Donkey Serum in PBS with
266 0.02% Polysorbate 20) in the circle of the slide and incubate at 4 ˚C overnight.
267
268 NOTE: To judge the substage of meiotic spermatocytes, SYCP3 was detected, which is a marker
269 of meiotic chromosome axes, and γH2AX, which is a marker of DNA damage response. (For the
270 antibody working concentration, please refer to the **Table of Materials**).
271
272 6.8. Tap off the primary antibody solution from the slides.
273
274 6.9. Drop 50 μL of PBS in the circle of the slide and tap off. Repeat one time.
275
276 6.10. Add secondary antibody solution (dilute secondary antibodies in PBS with 0.02%
277 Polysorbate 20) in the circle of the slide and incubate at room temperature for 1 h in dark.
278
279 6.11. Drop 50 μL of PBS in the circle of the slide and tap off. Repeat one time.
280
281 6.12. Add 50 μL of DAPI (stock concentration is 0.1 μg/mL) stain for 5 min and tap off.
282
283 6.13. Add 1-2 drops of mounting media on the slides. Carefully cover the mounting media with a
284 cover glass and gently press the cover glass to remove extra mounting media and air bubble. The
285 slides are ready for microscopy evaluation.
286
287 **REPRESENTATIVE RESULTS:**
288 A representative result of this sorting protocol is shown in **Figure 3**. The total sorting time of two
289 testes (one mouse) is usually around 3 hours, which is dependent on the concentration of cell
290 suspension and the sorting speed. After sorting, the purity of spermatocytes is confirmed by
291 immunostaining of SYCP3 and rH2AX (**Figure 3A**). The representative purity of sorted L/Z, P, D
292 spermatocyte fractions are around 80.4%, 90.6%, and 87.6%, respectively (**Figure 3C**). We have
293 determined the substages based on the criteria we published previously[19]. Briefly, in the
294 leptotene and zygotene stage, synapsis between homologous chromosomes is incomplete, which
295 is indicated by thin threads of SYCP3 staining. Broad γH2AX domains are observed throughout
296 the nuclear chromatin due to programmed DNA double-strand breaks. In the pachytene stage,
297 homologous chromosomes have completely synapsed, and γH2AX specifically accumulates on
298 the sex chromosomes. In the diplotene stage, homologous chromosomes progressively undergo
299 desynapsis. The purity of RS is confirmed by nucleus staining with DCV (**Figure 3B**). RS can be
300 precisely judged with DNA staining: a unique DAPI-intense chromocenter surrounded by
301 euchromatin; or combine with specific markers, such as Sp56 that is expressed within the
302 developing acrosomal granule of spermatid and histone variant H1T that is highly expressed in
303 nucleus after the mid-pachytene stage (**Figure 3B**).
304
305 RS purity is around 90.1% after sorting (**Figure 3C**). The sample size of purity analysis is over 1,000
306 cells for each experiment; the purity of L/P and D populations is averaged from 6 independent
307 experiments; the purity of P and RS populations is averaged from 3 independent experiments.
308 The viability of these isolated cells is usually over 95% (**Figure S1**). The total yield of each fraction

309   from a single adult mouse is estimated and listed in Fig 3C, which provides sufficient cells for
310   various downstream analyses. Recently, we have used this protocol to isolate wild-type
311   pachytene spermatocytes for ChIP-seq analysis[20,21].
312
313   **FIGURE AND TABLE LEGENDS:**
314   **Table 1: Reagent Recipe.** The dissociation buffer must be prepared right before use. Prewarm
315   DMEM before starting dissection. The enzyme stocks can be prepared any time before the
316   experiment and stored at -20 ˚C. FACS buffer needs to be vacuum-filtered and stored at 4 ˚C;
317   prewarm to room temperature before use.
318
319   **Figure 1: Workflow of murine spermatogenic cells isolation on DCV-based sorting.** This image
320   illustrates the general procedure, from tissue dissociation to FACS sorting, to harvesting of
321   isolated spermatogenic cells within one day.
322
323   **Figure 2: FACS analysis of adult murine testicular cells based on DCV fluorescence and light**
324   **scattering.** (**A**) Acquired unstained cells in the first decade of a DCV-blue histogram plot (left side
325   of the red bar). (**B**)(**C**) Debris and non-single cell excluded by light scattering. (**D**) Unstained cell
326   and side population exclusion based on DCV fluorescence. (**E**) DNA content determination based
327   on DCV-blue fluorescence. Left peak (green) and right peak (pink) correspond to 1C and 4C
328   populations. (**F**) Gating on 1C and 4C testicular populations based on DCV-blue/DCV-red
329   fluorescence. (**G**) Precise gating on 4C testicular populations. (**H**) Back-gating of Gate 4C from the
330   DCV plot on an FSC/BSC plot. Based on regions of minimal overlap on the FSC/BSC plot, the L/Z,
331   P, and D gates are created to enrich their respective spermatocyte populations. (**I**) Color dot plot
332   showing the L/Z, P, and D populations are in continuous order within Gate 4C. (**J**) Back-gating of
333   Gate 1C from the DCV plot on an FSC/BSC plot. RS gate was created to enrich round spermatid
334   population with uniform size, resulting in greater purity of populations during sorting.
335
336   **Figure 3: Representative result images and statistics of spermatogenic cells obtained from**
337   **sorting.** (**A**) Immunofluorescence characterization of sorted spermatocytes. Upper panel: DCV
338   staining showing nucleus pattern of the live spermatocytes right after sorting; L/Z
339   (leptotene/zygotene), P (pachytene), and D (diplotene). Lower panel: Confirmation of meiotic
340   substages for each population by immunostaining for SYCP3 (green) and γH2AX (red). (**B**)
341   Representative DCV image showing nucleus pattern of RS. Scale bars: 50 μm (upper panels), and
342   10 μm (lower magnified panels). Right Panels: Immunofluorescence confirmation of round
343   spermatids stained with Sp56 and H1T. (**C**) The purity of L/Z, P, and D were confirmed by
344   immunostaining, sample size was over 1,000 cells for each independent experiment, in total 6
345   independent experiments. The RS purity was confirmed by nucleus staining with a total of 3
346   independent experiments. The total cell number of the testicular cell suspension from one 8-
347   week-old WT B6 mouse was around 100 million cells before sorting.
348
349   **Figure S1: The viability of isolated pachytene spermatocytes.** A representative image shows the
350   cell viability of isolated pachytene spermatocytes (Red: PI; Blue: DCV). PI could not be combined
351   with DCV during sorting. However, under microscope, the DCV-red signal was quite low;

352 therefore, PI-positive dead cells were easily distinguished from other live cells. The viability is
353 usually over 95%. Scale bars: 10 μm.
354
355 **Figure S2: Incomplete dissociation or debris disturbs gating.** The A population (red circle)
356 contains debris and polymer of spermatids (indicated by arrow). The bigger A population will
357 cross with B population (yellow circle) and eventually contaminate the 4C population. Scale bars:
358 200 μm.
359
360 **DISCUSSION:**
361 Here we present a practical and simple protocol to isolate subpopulations of spermatocytes and
362 spermatids from a single adult male mouse. To ensure the reproducibility of this protocol, there
363 are some critical steps that need attention. Before enzyme digestion, wash step aims to remove
364 interstitial cells; after digestion, this step helps to remove spermatozoa and debris.
365 Washing/centrifuging 3 times is important. In our dissociation buffer recipe, the combination of
366 several different enzymes facilitates the dissociation of testes into the single-cell suspension
367 without excessive cell damage. Gently pipetting to avoid causing air bubbles also helps to protect
368 cell integrity. Please check the cell suspension under a microscope after dissociation to make sure
369 the suspension achieves single-cell level. Incomplete dissociation or debris contamination from
370 excessive digestion will affect the purity of sorted cells; as shown in **Figure S2**, the upright
371 population contains debris and tetramer of spermatids. DCV staining requires incubation in the
372 dark and no washing afterwards.
373
374 To troubleshoot potential difficulties on gating and back-gating of spermatocyte subpopulations,
375 as an option for optimization, we recommend using a synchronized wild type mouse to help
376 locate a specific stage of spermatocytes[22-24]. It is also worth noting that some knockout mouse
377 strains with spermatogenesis arrest phenotypes may have uncommon DCV profiles because they
378 are missing some subpopulations. Proper wildtype control is strongly recommended in this case.
379 In addition, this protocol can be potentially applied to adult mice of any age. However, the age
380 of the experimental mouse could be a confounding factor due to the variable proportion of germ
381 cells.
382
383 Over the years, several protocols to purify germ cells have been developed. As one of the most
384 popular methods, STA-PUT velocity sedimentation separates germ cells by the BSA gradient and
385 provides a good yield of intact germ cells[6,7]. However, STA-PUT not only requires special devices
386 that may not be readily available to many laboratories but is also time-consuming and labor-
387 intensive to conduct in a cold room at 4 °C. Unlike STA-PUT, which is suitable for large-scale
388 separation, this FACS-based method could provide high purity and precise fraction for a small-
389 scale experiment. A large-scale sorting using our protocol is possible but will prolong the sorting
390 time significantly and may compromise cell viability. Therefore, STA-PUT is still a practical option
391 when a large number of cells is needed[5,25,26].
392
393 In comparison with the previous FACS method based on Ho342 dye staining[8-11], our protocol
394 utilizes DCV, which has a broader excitation spectrum and can be applied to most current FACS
395 sorters equipped with a UV or 405 nm violet laser[18]. Although there is another protocol using

396 DCG[14-17], the difference between our protocol and the DCG protocol is that our DCV protocol uses
397 two-dimensional separation with DCV blue and DCV red, mimicking the advantage of the Ho342
398 protocol. With this advantage, our DCV protocol allows us to isolate highly enriched germ cells
399 from adult testis. The DCG protocol does not employ two-dimensional separation and is
400 recommended for isolation of germ cells from juvenile mice. The two-dimensional separation can
401 have better resolution to separate the substages of spermatocytes. However, our method is still
402 incapable of isolating leptotene and zygotene spermatocytes separately, as well as "2C" cell types
403 including spermatogonia, preleptotene spermatocytes, and secondary spermatocytes.
404
405 Since the wide emission spectrum of DCV stain causes leaking to other channels, most of the cell
406 viability dyes like PI and 7AAD cannot be combined due to false positive signals. Other cell viability
407 dyes with emission in far-red or near-infrared channels might be worth trying in the future. But
408 in our experience, sorted cells usually show ≥ 95% viability after 2 hours of FACS sorting (**Figure**
409 **S1**), which is sufficient for downstream analysis.
410
411 Sorted cells obtained from our procedure can be used for various downstream experiments,
412 including next-generation sequencing analysis (RNA-seq, ATAC-seq, and ChIP-seq). Cells obtained
413 here can also be used for short-term culture[27]. In conclusion, we provide a simple but efficient
414 protocol including a one-hour single-cell suspension preparation procedure and the detailed
415 gating strategy for FACS based on DCV dye staining, which is suitable for small-scale
416 spermatogenic cell isolation and can be quickly adopted by many investigators, even flow
417 cytometry beginners.
418

432 **DISCLOSURES:**
433 The authors declare that they have no competing interests.
434

435 **REFERENCES:**
436 1. Griswold, M. D. Spermatogenesis: The Commitment to Meiosis. *Physiological Reviews.* **96**
437 (1), 1-17 (2016).
438 2. Yoshida, S. Mouse Spermatogenesis Reflects the Unity and Diversity of Tissue Stem Cell
439 Niche Systems. *Cold Spring Harbor Perspectives in Biology.* a036186 (2020).

440   3. Rathke, C., Baarends, W. M., Awe, S., Renkawitz-Pohl, R. Chromatin dynamics during
441   spermiogenesis. *Biochimica et Biophysica Acta.* **1839** (3), 155-168 (2014).
442   4. Maezawa, S. et al. SCML2 promotes heterochromatin organization in late spermatogenesis.
443   *Journal of Cell Science.* **131** (17), jcs.217125 (2018).
444   5. Maezawa, S., Yukawa, M., Alavattam, K. G., Barski, A., Namekawa, S. H. Dynamic
445   reorganization of open chromatin underlies diverse transcriptomes during spermatogenesis.
446   *Nucleic Acids Research.* **46** (2), 593-608 (2018).
447   6. Bellve, A. R. Purification, culture, and fractionation of spermatogenic cells. *Methods in*
448   *Enzymology.* **225**, 84-113 (1993).
449   7. Bryant, J. M., Meyer-Ficca, M. L., Dang, V. M., Berger, S. L., Meyer, R. G. Separation of
450   spermatogenic cell types using STA-PUT velocity sedimentation. *Journal of Visualized*
451   *Experiments.* (80), e50648 (2013).
452   8. Gaysinskaya, V., Bortvin, A. Flow cytometry of murine spermatocytes. *Current Protocols in*
453   *Cytometry.* **72** 7.44.41-47.44.24 (2015).
454   9. Bastos, H. et al. Flow cytometric characterization of viable meiotic and postmeiotic cells by
455   Hoechst 33342 in mouse spermatogenesis. *Cytometry Part A.* **65** (1), 40-49 (2005).
456   10. Getun, I. V., Torres, B., Bois, P. R. Flow cytometry purification of mouse meiotic cells.
457   *Journal of Visualized Experiments.* (50), e2602 (2011).
458   11. Gaysinskaya, V., Soh, I. Y., van der Heijden, G. W., Bortvin, A. Optimized flow cytometry
459   isolation of murine spermatocytes. *Cytometry Part A.* **85** (6), 556-565 (2014).
460   12. Romer, K. A., de Rooij, D. G., Kojima, M. L., Page, D. C. Isolating mitotic and meiotic germ
461   cells from male mice by developmental synchronization, staging, and sorting. *Developmental*
462   *Biology.* **443** (1), 19-34 (2018).
463   13. Lam, K. G., Brick, K., Cheng, G., Pratto, F., Camerini-Otero, R. D. Cell-type-specific genomics
464   reveals histone modification dynamics in mammalian meiosis. *Nature Communications.* **10** (1),
465   3821 (2019).
466   14. Geisinger, A., Rodríguez-Casuriaga, R. Flow Cytometry for the Isolation and Characterization
467   of Rodent Meiocytes. *Methods in Molecular Biology.* **1471**, 217-230 (2017).
468   15. da Cruz, I. et al. Transcriptome analysis of highly purified mouse spermatogenic cell
469   populations: gene expression signatures switch from meiotic-to postmeiotic-related processes
470   at pachytene stage. *BMC Genomics.* **17**, 294 (2016).
471   16. Rodríguez-Casuriaga, R. et al. Rapid preparation of rodent testicular cell suspensions and
472   spermatogenic stages purification by flow cytometry using a novel blue-laser-excitable vital dye.
473   *MethodsX.* **1**, 239-243 (2014).
474   17. Trovero, M. F. et al. Revealing stage-specific expression patterns of long noncoding RNAs
475   along mouse spermatogenesis. *RNA Biology.* **17** (3), 350-365 (2020).
476   18. Telford, W. G., Bradford, J., Godfrey, W., Robey, R. W., Bates, S. E. Side population analysis
477   using a violet-excited cell-permeable DNA binding dye. *Stem Cells.* **25** (4), 1029-1036 (2007).
478   19. Alavattam, K. G., Abe, H., Sakashita, A., Namekawa, S. H. Chromosome Spread Analyses of
479   Meiotic Sex Chromosome Inactivation. *Methods in Molecular Biology.* **1861**, 113-129 (2018).
480   20. Maezawa, S. et al. Super-enhancer switching drives a burst in gene expression at the
481   mitosis-to-meiosis transition. *Nature Structural & Molecular Biology.* accepted (2020).
482   21. Sakashita, A. et al. Endogenous retroviruses drive species-specific germline transcriptomes
483   in mammals. *Nature Structural & Molecular Biology.* accepted (2020).

484   22. Agrimson, K. S. et al. Characterizing the Spermatogonial Response to Retinoic Acid During
485   the Onset of Spermatogenesis and Following Synchronization in the Neonatal Mouse Testis.
486   *Biology of Reproduction.* **95** (4), 81 (2016).
487   23. Hogarth, C. A. et al. Turning a spermatogenic wave into a tsunami: synchronizing murine
488   spermatogenesis using WIN 18,446. *Biology of Reproduction.* **88** (2), 40, (2013).
489   24. Patel, L. et al. Dynamic reorganization of the genome shapes the recombination landscape
490   in meiotic prophase. *Nature Structural & Molecular Biology.* **26** (3), 164-174 (2019).
491   25. Alavattam, K. G. et al. Attenuated chromatin compartmentalization in meiosis and its
492   maturation in sperm development. *Nature Structural & Molecular Biology.* **26** (3), 175-184
493   (2019).
494   26. Adams, S. R. et al. RNF8 and SCML2 cooperate to regulate ubiquitination and H3K27
495   acetylation for escape gene activation on the sex chromosomes. *PLoS Genetics.* **14** (2),
496   e1007233 (2018).
497   27. Wiltshire, T., Park, C., Caldwell, K. A., Handel, M. A. Induced premature G2/M-phase
498   transition in pachytene spermatocytes includes events unique to meiosis. *Developmental*
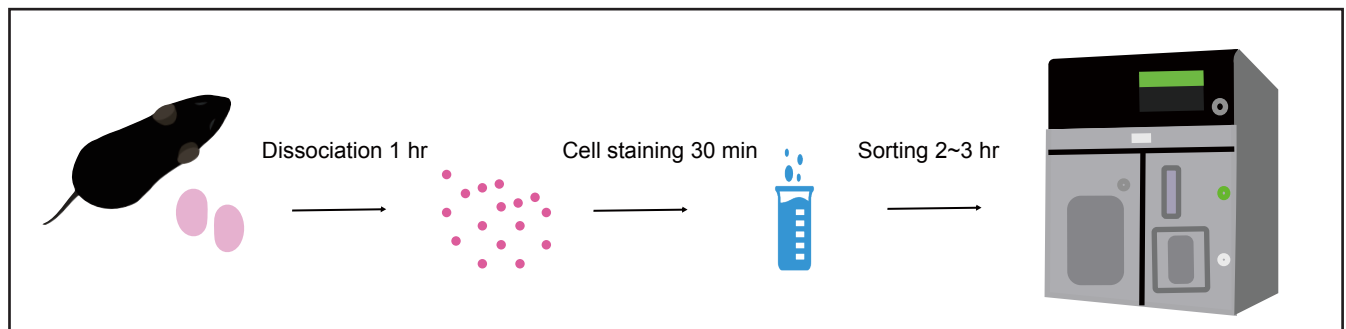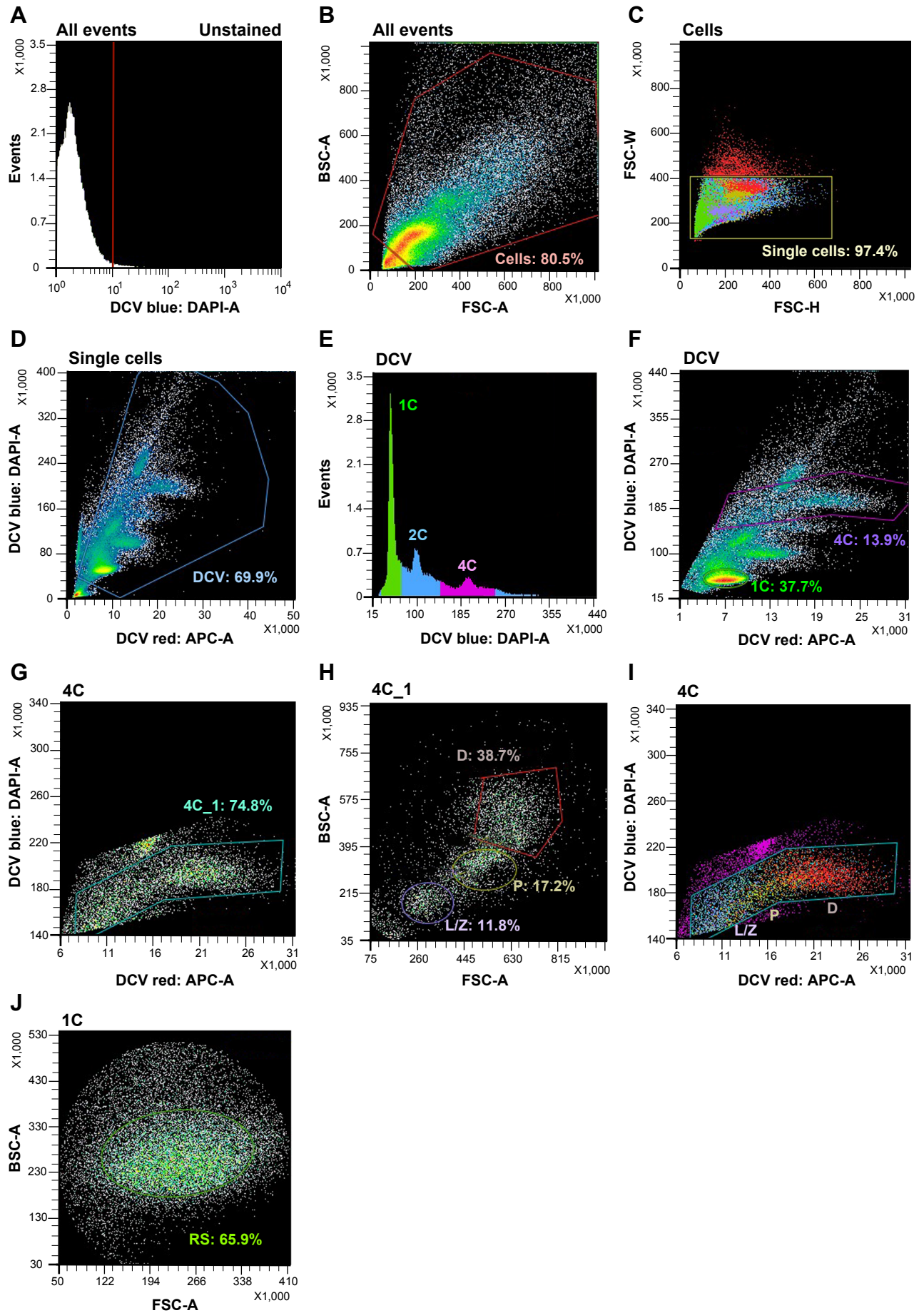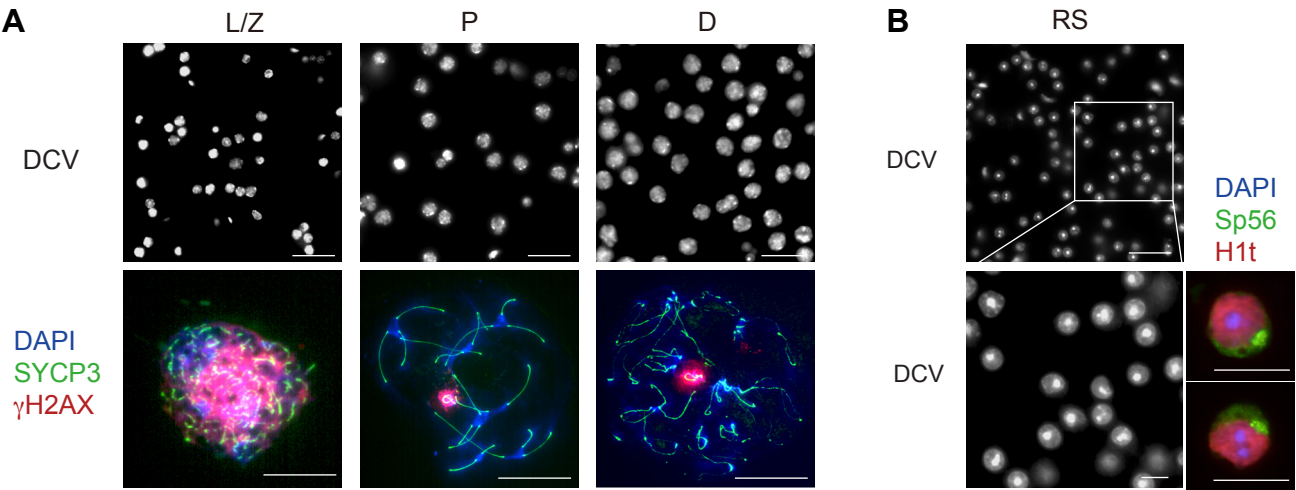499   *Biology.* **169** (2), 557-567 (1995).
500

Figure 1

Dissociation 1 hr   Cell staining 30 min   Sorting 2~3 hr

Figure 2
Click here to access/download;Figure;Yeh_Fig.2.pdf ⬇

Figure 3

A

L/Z   P   D

DCV

DAPI
SYCP3
γH2AX

B

RS

DCV

DAPI
Sp56
H1t

DCV

C

Isolated cell types	L/Z	P	D	RS
Purity (mean ± SD) %	80.4 ± 10.6	90.6 ± 4.88	87.6 ± 6.51	90.1 ± 7.22
Isolated cell numbers/ one adult mouse	100,000	140,000	300,000	2,000,000

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 ml tube	Watson	131-7155C	
100 mm Petri dish	Corning, Falcon	351029	
15 mL Centrifuge tube	Watson	1332-015S	
5 ml polystyrene tube with cell strainer snap cap (35 μm nylon mesh)	Corning, Falcon	352235	
50 mL Centrifuge tube	Watson	1342-050S	
60 mm Petri dish	Corning, Falcon	351007	
70 μm nylon mesh	Corning, Falcon	352350	
Cell sorter	Sony	SH800S	
Centrifuge			
Collagenase, recombinant, Animal-de	FUJIFILM Wako Pu	036-23141	
Collagenase, Type 1	Worthington	LS004196	
Cover glass	Fisher	12-544-G	
Cytospin 3	Shandon		
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Fisher	D1306	working concentration: 0.1 μg/mL
Dnase I	Sigma	D5025-150KU	
Donkey serum	Sigma	S30-M	
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	14190144	
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	11885076	
Fetal bovine serum (FBS)	Gibco	16000044	
Hostone H1T antibody	gift from Mary Ann Handel		1/2000 diluted
Hank's balanced salt solution (HBSS)	Gibco	14175095	
Hyaluronidase from bovine testes	Sigma	H3506-1G	
Phosphate buffered saline (PBS)	Sigma	P5493-4L	
Pipettemen			
ProLong Gold Antifade Mountant	Fisher	P36930	

rH2AX antibody	Millipore	05-635	working concentration: 2 µg/mL
Sperm Fertilization Protein 56 (Sp56) antibody	QED Bioscience	55101	working concentration: 0.5 µg/mL
Sterilized forceps and scissors			
Superfrost /Plus Microscope Slides	Fisher	12-550-15	
SYCP3 antibody	Abcam	ab205846	working concentration: 5 µg/mL
TWEEN 20 (Polysorbate 20)	Sigma	P9416	
Vybrant DyeCycle Violet Stain (DCV)	Invitrogen	V35003	
Water bath			

Response to Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

We used the file for revision.

2. Please address all specific comments marked in the manuscript.

**We addressed all specific comments in the text. Please see out response in the text.**

3. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.
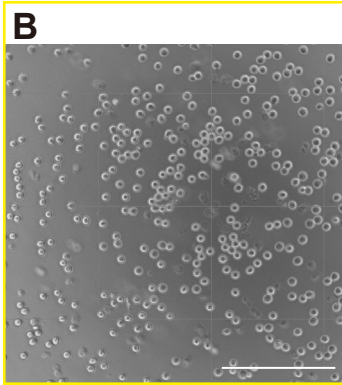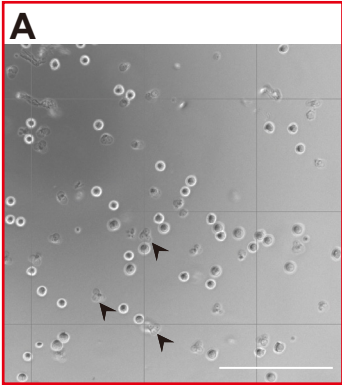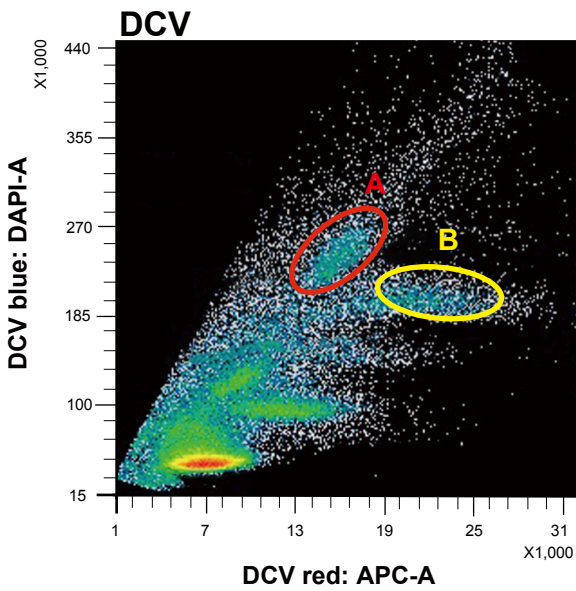
Done. It is now almost 2.75 page length.

4. Please reword lines 180-182, 184-186, 224-227, 270-272. Is figure 2 taken from previously published literature? If not please reword the legend.

We reworded the suggested sentences. Figure 2 is not taken from previous literature. We reworded the legend.

DCV
PI

Table 1
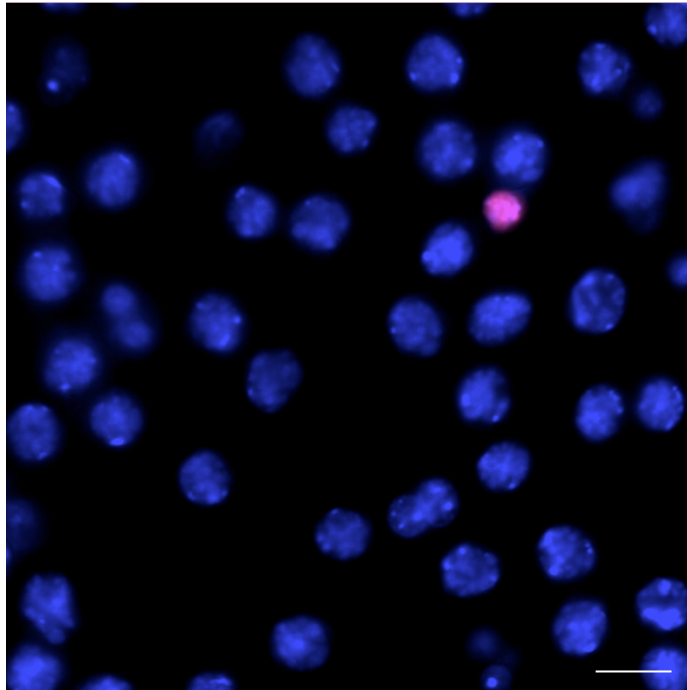
Reagent	Ingredient	Stock concentration (HBSS base)	Volume
Dissociation Buffer (DMEM base)	DMEM	-	2 ml
	FBS	-	40 ul
	Hyaluronidase	100mg/ml	30 ul
	DNase I	10 mg/ml	50 ul
	Collagenase Type I	100 mg/ml	40 ul
	Recombinant Collagenase	14000 unit/ml	100 ul
FACS buffer (PBS base)	PBS	-	980ml
	FBS	-	20ml