Chapter 26

The Sister-Chromatid Exchange Assay in Human Cells

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Abstract

The semiconservative nature of DNA replication allows the differential labeling of sister chromatids that is the fundamental requirement to perform the sister-chromatid exchange (SCE) assay. SCE assay is a powerful technique to visually detect the physical exchange of DNA between sister chromatids. SCEs could result as a consequence of DNA damage repair by homologous recombination (HR) during DNA replication. Here, we provide the detailed protocol to perform the SCE assay in cultured human cells. Cells are exposed to the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) during two cell cycles, resulting in the two sister chromatids having differential incorporation of the analog. After metaphase spreads preparation and further processing, SCEs are nicely visualized under the microscope.

Key words Sister-chromatid exchange (SCE), 5-Bromo-2′-deoxyuridine (BrdU), Homologous recombination (HR), Human cells, DNA replication

1 Introduction

Sister-chromatid exchange (SCE) assay is a well-established technique to detect the exchange of DNA between sister chromatids. In order to visually detect these events in metaphase chromosomes, chromatids have to be differentially stained and this could be accomplished taking advantage of the semiconservative mechanism of DNA replication. This technique was initially performed culturing cells in medium supplemented with tritium-labeled thymidine for one cell cycle followed by incubation in radioactive-free medium for an additional cell cycle [1]. However, the use of radiolabeled nucleotides is per se a source of DNA breaks, as a consequence of which it increases SCEs. Indeed, SCEs have been shown to be induced by DNA damaging agents, and for this reason, the SCE assay has been employed as a method to analyze the potential mutagenicity of different compounds [2]. Repair of double-strand breaks (DSBs) during replication by homologous recombination (HR) is considered to be the main source of SCEs [3, 4]. In cycling cells, SCEs occur spontaneously in association with DNA

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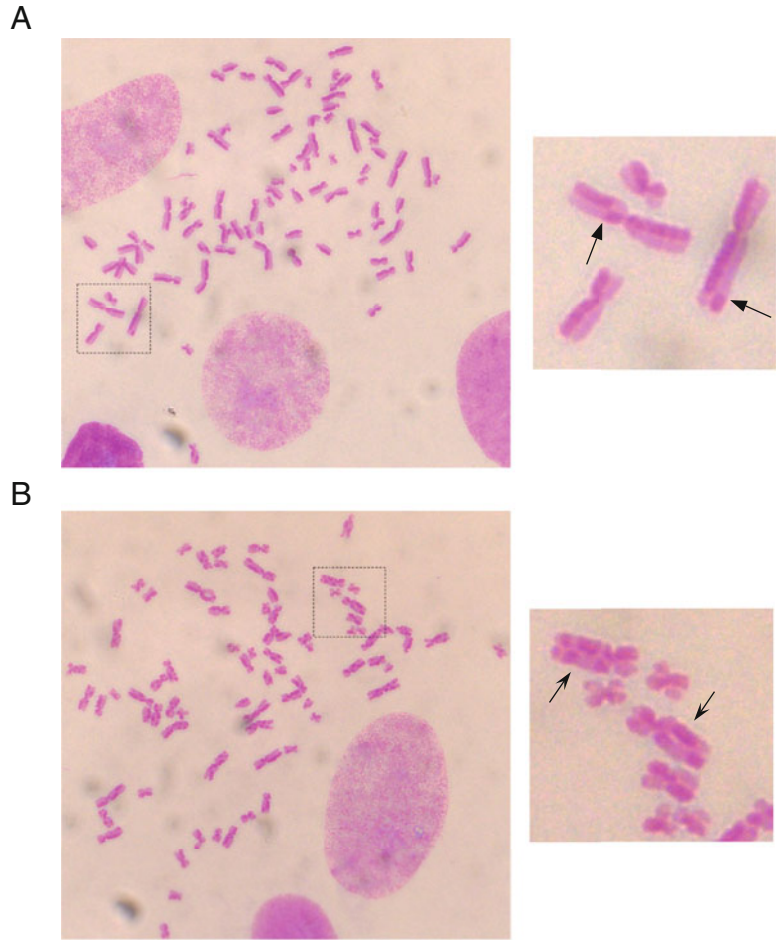

Fig. 1 Example of metaphase spreads to analyze SCE. (**a**) Untreated HeLa cells. Inset magnifies four chromosomes to better appreciate the differential stained chromatids; arrowheads point out SCEs. (**b**) HeLa cells exposed to 3 nM of camptothecin during 45 h. Inset magnifies chromosomes with many SCEs also named "harlequin" chromosomes, pointed out by arrows. The shown images have been captured using a Leica AF600 upright microscope, objective HCX PL APO CS 100×/1.4 OIL, equipped with a Leica DC350 FX camera and using the Leica Application Suite LAS v3.8

replication and upon replication fork breakage, at an estimated frequency of 3–4 events per cell cycle (Fig. 1a) [5]. The use of genotoxic treatments that produce replication-dependent DNA breaks, such as camptothecin (CPT), dramatically increases the levels of SCEs accumulated per cell and per chromosome (Fig. 1b). Chromosomes with many SCEs are named "harlequin" for their peculiar phenotype (Fig. 1b, inset). Harlequin chromosomes are also a distinctive trait of cells from Bloom's syndrome

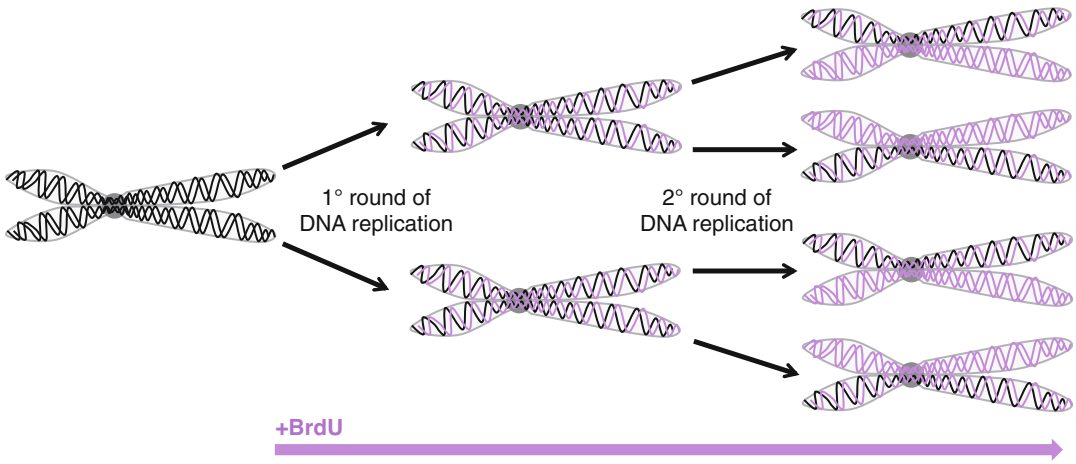

Fig. 2 Schematic representation of the process leading to the differential incorporation of BrdU in sister chromatids

patients in which the mutation of the Bloom helicase gene confers genome instability and a striking increase in SCE [6]. A crucial improvement of this technique derived from the finding that DNA, after 5-bromo-2′-deoxyuridine (BrdU) incorporation followed by fluorescence staining with Hoechst and light exposition, is much weakly stained by Giemsa [7]. Therefore, chromatids with different amount of incorporated BrdU stained by Giemsa will result darker or lighter accordingly. Hence, the following protocol employs this method to differentially label the two chromatids to be able to detect SCEs.

Human cells are fed during two cell cycles with medium supplemented with BrdU. During the first round of replication, each chromosome duplicates in two chromosomes having both chromatids half BrdU substituted. Following the second round of DNA replication, each chromosome gives rise to two chromatids, one fully BrdU substituted and the other half BrdU substituted (Fig. 2). At this point, the sister chromatids have different amount of incorporated BrdU that, after the subsequent procedure, will stain differentially. In order to enrich for metaphase cells, the cells are then treated with Colcemid, an inhibitor of microtubule polymerization and mitotic spindle formation that arrests cells in metaphase [8]. The timeline of this procedure is depicted in Fig. 3. Subsequent to hypotonic treatment and fixation, the suspension of swollen cells is dropped onto microscope slides leading to chromosomes spreading. Staining with Hoechst 33258 and exposure to UVA cause BrdU-based DNA photodegradation that produce different affinities to Giemsa stain.

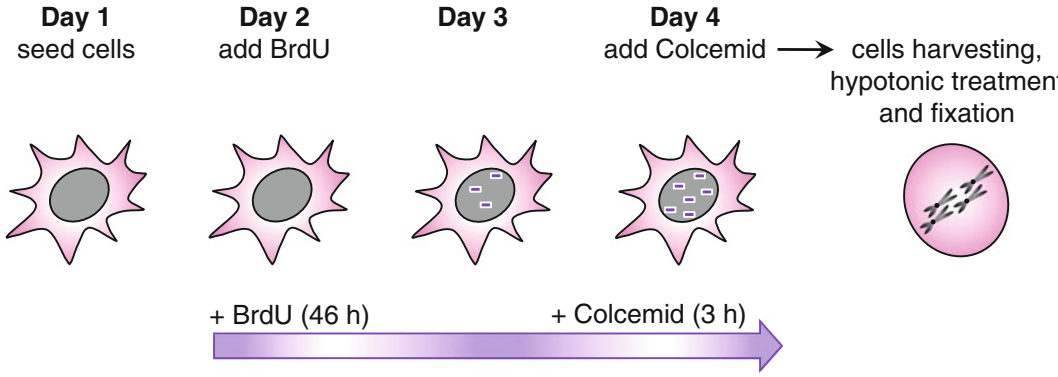

Fig. 3 Cell culture timeline to obtain metaphase cells with differential stained chromatids

2 Materials

2.1 BrdU Incorporation and Cells Harvesting for Metaphase Spread

1. 10 cm tissue culture plates.
2. HeLa or U2OS cell lines (*see* **Note 1**).
3. Tissue culture incubator.
4. Laminar flow hood and standard tissue culture setup, including serological pipettes, pipettor, micropipettes, vacuum aspiration apparatus, and inverted microscope.
5. Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 60 μg/ml penicillin, and 0.25 μg/ml amphotericin B.
6. Ca^{2+}/Mg^{2+}-free PBS.
7. Trypsin-EDTA.
8. 5-Bromo-2′-deoxyuridine (BrdU) (Sigma).
9. KaryoMAX™ Colcemid™ Solution in PBS (Gibco).
10. Hypotonic buffer (KCl 0.075 M).
11. Fixative (3 volumes of methanol: 1 volume of acetic acid, freshly prepared).
12. 15 ml centrifuge tubes.
13. 1.5 ml microcentrifuge tubes.
14. Aluminum foil.
15. Tabletop centrifuge (Beckman Allegra X-12 Tabletop Centrifuge) and microcentrifuge.
16. 37 °C water bath.

2.2 Preparing Metaphase Spreads

1. Frosted microscope slides.
2. Pencil.

3. 70% ethanol.
4. 45% acetic acid solution.
5. P200 pipette.
6. Glass Coplin jar.
7. Beaker 500 ml.
8. 65 °C incubator.

2.3 Chromatid Differential Staining

1. Phosphate buffer tablets pH 6.8—for preparing buffer solution according to WEISE (Merck). Dissolve 1 tablet in 1 l of distilled water.
2. Hoechst 33258, 20 mM solution in water (AnaSpec).
3. Saline-Sodium Citrate (SSC) 2× buffer.
4. Methanol (Sigma).
5. Modified Giemsa stain (Sigma).
6. UV lamp (BS03 UV irradiation chamber; Dr. Gröbel UV-Elektronik GmbH).
7. 60 °C incubator.
8. Plastic tray (to place the microscope slides).
9. 24 × 50 mm coverslip, thickness should be 0.17 mm or less (optional).
10. Synthetic resin mounting medium (optional).

2.4 Chromosome Spreads Scoring by Microscope and Sister-Chromatid Analysis

1. Bright-field microscope with at least a 40× dry objective and a 100× high-quality oil-immersion objective.
2. Immersion oil.
3. High-resolution microscope digital camera.
4. Computer and microscope software for image acquisition.
5. ImageJ software [9].

3 Methods

3.1 BrdU Incorporation and Cells Harvesting for Metaphase Spread

1. Plate cells in complete DMEM in 10 cm plates in order to have them 20% confluent the following day (*see* **Note 2**).
2. 18–20 h after cell seeding, replace the medium with fresh complete DMEM supplemented with BrdU at final concentration of 10 μM. Wrap the plates with aluminum foil before returning them to the incubator (*see* **Notes 3** and **4**).
3. 43 h after BrdU addition, to arrest cells in metaphase add Colcemid to the final concentration of 0.1 μg/ml directly to the medium, wait for 3 more hours and proceed to harvest cells for metaphase spreads (*see* **Note 5**).

4. Collect the supernatant in a 15 ml centrifuge tube, wash cells with 3 ml of Ca^{2+}/Mg^{2+}-free PBS and collect the PBS in the same centrifuge tube (*see* **Note 6**). Add 1 ml of trypsin-EDTA swirling the plate to ensure that it spreads covering all the surface and return the plate to 37 °C tissue culture incubator for approximately 3–5 min. When cells are clearly starting to detach, gently tap the border of the plate and looking the plate from the bottom check that cells are evenly detaching from all the surface of the plate. Add 3 ml of the medium containing FBS plus PBS previously collected in the centrifuge tube and immediately pipette the cell suspension up and down until obtaining a single-cell suspension (*see* **Note 7**). Collect cells into the same centrifuge tube and centrifuge them for 5 min at 250 × *g* at room temperature (RT).
5. Remove supernatant by vacuum aspiration and wash cells with 10 ml of PBS (*see* **Note 8**). Centrifuge for 5 minutes at 250 × *g* at RT to pellet cells down.
6. Remove supernatant by vacuum aspiration but leave approximately 200 μl of PBS to resuspend the pellet thoroughly by flicking.
7. Hypotonic treatment. Add 1 ml of pre-warmed (37 °C) KCl 0.075 M and pipette 2–3 times with a P1000 pipette. Add additional 9 ml of pre-warmed KCl 0.075 M, mix by inversion and incubate at 37 °C in a water bath for 10 min (*see* **Note 9**).
8. Add 300 μl of fixative solution dropwise, gently mix by inverting the tube and centrifuge for 5 min at 250 × *g* at RT (*see* **Note 10**).
9. Remove supernatant by vacuum aspiration but leave approximately 200 μl of volume to resuspend cells by flicking. Wash with 10 ml of fixative and repeat this **step 4** more times.
10. Resuspend the pellet in 500 μl of fixative solution and transfer to a 1.5 ml microtube. The protocol may be stopped at this point and the cells stored at −20 °C indefinitely.

3.2 Preparing Metaphase Spreads

1. Warm up cell suspension at room temperature before starting. If samples have been stored for more than 1 day before starting this procedure, centrifuge the sample and resuspend cells in fresh fixative.
2. Clean microscope frosted slides with 70% ethanol, 1 for each experimental condition, and label them with a pencil on the frosted side.
3. For this procedure refer to Fig. 4. Hold a microscope slide by the side of the frosted edge, parallel to the bench and above the 500 ml beaker that will serve to collect waste. Pour 45% acetic acid as much as to cover the slide, you have to create a layer of

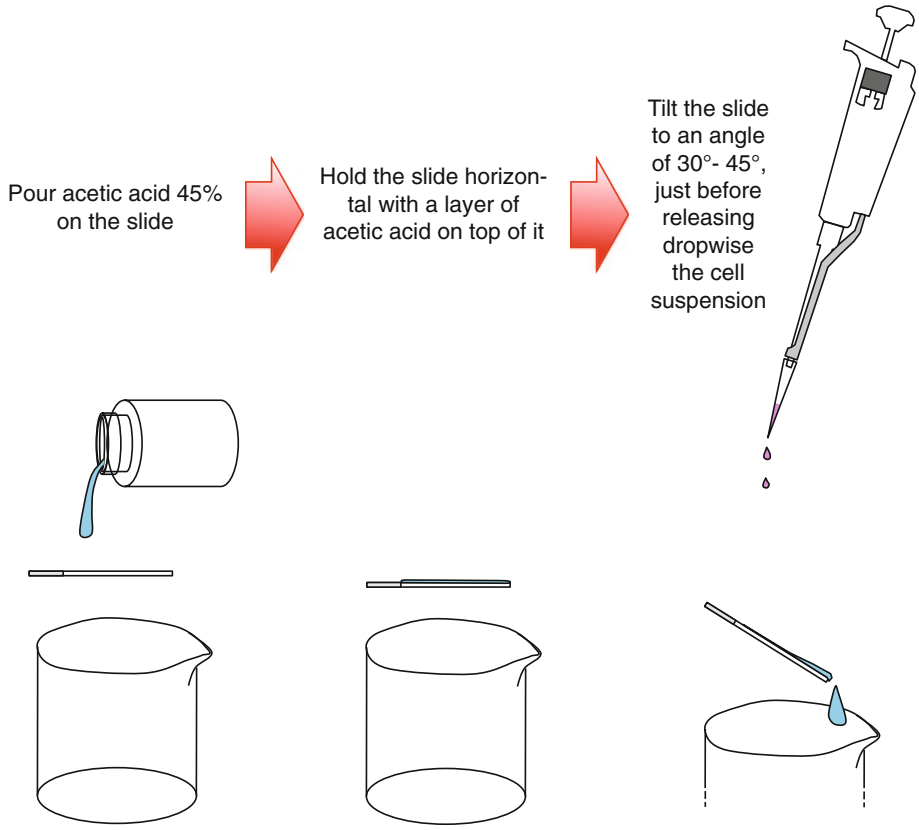

Fig. 4 Schematic representation of the procedure to spread cells on microscope slides

this solution over the slide. Resuspend cell suspension in fixative just before its use, collect 100–150 µl with a P200 pipette and release dropwise on the microscope slide. Just before the dropping of cell suspension onto the slide, incline the slide to 30–45° angle letting the acetic acid slide away. Drops of cells suspension have to fall from a height of approximately 10–15 cm above the slide (*see* **Note 11**).

4. Tap the edge of the slide opposite to the frosted edge onto paper towel to remove the excess of liquid. Let the slides air dry in horizontal position (*see* **Note 12**).
5. Before staining, incubate the slides at 65 °C overnight or alternatively store them for 3 days at room temperature (aging procedure).

3.3 Sister-Chromatid Differential Staining

1. Incubate the slides with Hoechst 33258 at 20 µg/ml (freshly prepared by diluting the 20 mM solution in distilled water) for 20 min in dark (*see* **Note 13**).
2. Wash with distilled water.

3. Place the slides upward horizontally on a tray and fill with SSC 2× buffer up to cover them by 1–2 mm. Expose to UVA irradiation during 1 h.
4. Change the buffer with SCC 2× pre-warmed at 60 °C and incubate for 20 min at 60 °C (*see* **Note 14**).
5. Wash with distilled water.
6. Incubate 5 min in methanol.
7. Incubate 20 min in Giemsa staining solution (Giemsa diluted 1:20 in Weise buffer).
8. Wash one or two times quickly with Weise buffer and let the slides air dry in a vertical position (*see* **Note 15**). Store the slides overnight at RT to be sure they completely dry.
9. Metaphase chromosomes could be scored directly under the microscope or the slides could be mounted using a synthetic resin mounting medium (DPX, Permount, Cytoseal 60 or similar) and 24 × 50 mm coverslips. Soak slides in xylene before applying the mounting media as a longitudinal line on the center of the coverslip. Starting from one edge carefully let the coverslip adhere to the slide and the mounting media spread homogenously without trapping air bubbles. Remove the excess of mounting medium and wait until it completely dries before microscope exploration.

3.4 Chromosome Spreads Scoring by Microscope and Sister-Chromatid Analysis

1. Using a bright-field microscope, score for metaphases by 40× dry objective.
2. Once encountered an informative metaphase, change to 100× oil-immersion objective and capture an image (*see* **Notes 16** and **17**).
3. Score and record images of at least 20 metaphases.
4. To analyze the SCEs, use the "cell counter" plugin of the software ImageJ. Count the number of chromosomes per metaphase and the number of SCE per chromosome to obtain the number of exchanges per chromosome.

4 Notes

1. Here, we describe the optimized conditions for these two cell types. However, thanks to the detailed explanation of every step of the procedure, this assay could be performed with any kind of proliferating cell lines, once the cell line-dependent parameters have been adapted accordingly to the specific cell features.
2. The optimal seeding density of cells is important. Cells have to be in exponential grow during BrdU incorporation and at the

moment of Colcemid treatment. Only exponentially growing cells would have two rounds of DNA replication and afterwards would efficiently progress to mitosis. If cells get too close to confluency it will result in incomplete chromatid labeling and/or poor metaphases recovery.

3. The best option is to seed cells in late afternoon and feed them with BrdU the following morning. For fast proliferating cells, waiting the least possible time between the seeding and the start of the supplementation with BrdU is helpful in order to have cells still in exponential growth at the moment of Colcemid treatment. In case the cell type needs more time to adhere properly and start growing after trypsinization and plating, it might be useful to seed the cells in half of the usual volume (e.g., in 5 ml instead than 10 ml for a 10 cm plate) and the following day add 1 volume of medium containing 2× BrdU.
4. Minimize light exposure. BrdU is light sensitive, and cells incorporating BrdU are more susceptible to light-induced DNA breaks since light exposure induces photolysis of DNA at sites of BrdU incorporation.
5. These time points are optimal for HeLa, U2OS, and all the cell types with similar growth rate. In case of a slower proliferating cell type, both BrdU and Colcemid incubations could be lengthened or, in the opposite situation that cells proliferate faster, they could be shortened. At the moment of Colcemid treatment, cells should be approximately 70–80% confluent to ensure their progression through mitosis and the recovery of a sufficient number of metaphases.
6. Adherent cells such as HeLa or U2OS round up and detach (or loosen their adherence) from the plate surface when undergoing mitosis, recovering the supernatant and the PBS wash is a way to recover them all.
7. Single-cell suspension is important to distinguish efficiently single metaphases. Using small volume, such as 4 ml in this case, as well as pipetting straight after addition of FBS-containing medium to trypsin-detached cells, helps avoid the formation of cellular clumps. In the case that more than 1 plate has to be trypsinized, add FBS-containing medium to neutralize trypsin and pipette cell suspension one by one. If plates are numerous you could return most of them to room temperature a bit before cells are completely detached.
8. To ease the resuspension of the cells, it is better to add the first 1 ml of PBS, resuspend them by pipetting and then add the remaining 9 ml of PBS and mix the tube by inversion. Homogenous cells resuspension works better in smaller volumes.
9. The hypotonic treatment has to be adjusted to cell types. Too gentle treatment will result in metaphases too close,

chromosome crowded and overlapping and cytoplasm visible on the background. Too harsh condition will result in disrupted metaphases that could culminate in a "chromosomes soup" and in the impossibility to recognize individual metaphases. The condition described here works well for HeLa and U2OS cells.

10. After hypotonic treatment, the cell pellet should have doubled the size and should appear translucent since swollen cells have increased their water content.
11. At this point, it is possible to check cell density. In case the concentration is too high (cells and metaphase overlaps) or too low (too few metaphases to analyze), the sample could be diluted with fixative or concentrated after centrifugation, respectively .
12. The time that a slide takes to dry is a critical parameter because it influences the good spreading of chromosomes and it depends on environmental temperature and humidity. If the conditions are optimal, the slide will take 30–45 s to dry. In case it takes longer, it could be waved in the air. If, on the contrary, the slide dries too fast, it could be placed to dry on the top of a wet paper towel covered with a box lid in order to create a humid chamber.
13. This incubation could be performed in Coplin jar or, alternatively, to minimize the use of Hochest 33258, slides could be placed upward horizontally on a tray and 2–2.5 ml of the solution could be applied on the top of the entire slide.
14. From now on it is convenient to use a glass Coplin jar.
15. At this point, it is worthwhile to check by microscope using a dry 40× objective whether the staining worked fine. If chromosomes stained too dark, perform another wash with Weise buffer. If chromosomes are too lightly stained, repeat the staining with Giemsa staining solution for additional 20 min, wash quickly with Weise buffer and air dry.
16. A metaphase could be considered informative if the chromosomes display the differential staining of chromatids (may happen that not all the cells underwent two rounds of DNA replication), they are well separated (not overlapping) and clearly belonging to the same metaphase cell.
17. Good optics is very important in order to resolve the differentially stained chromatids and the exchanges of DNA between them. The 100× oil-immersion objective should have a numerical aperture of 1.4. Furthermore, closing the diaphragm aperture helps to increase the contrast and to get a clearer image.

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