

PKH26 红色荧光细胞连接试剂盒

PKH26 Red Fluorescent Cell Linker Kit

产品描述

PKH26 荧光细胞连接试剂盒采用本公司专利膜标记技术，能够将带有较长脂质尾巴的黄色-橙色荧光染料结合到细胞膜脂质区域上。染色方式依赖于细胞与膜的类型，主要用于细胞体外标记、体外细胞增殖研究及体内外细胞示踪研究。试剂盒中提供染色过程中所需的溶剂（稀释液 C），该溶剂可以在染色过程中，增加染料溶解度和染色效率，同时维持细胞活力。稀释液 C 与哺乳动物细胞等渗，且不含去垢剂或有机溶剂，也不含生理盐水和缓冲盐。根据细胞类型及标记后细胞膜内在的变化，标记后的细胞表面会由均一透亮变得有点状凸起或补丁状。但在生理范围内，PKH26 荧光不受 pH 的影响，每个细胞的荧光强度与染料标记位置无关。

PKH26 荧光在黄色-橙色区域 ($\lambda_{ex}=551\text{ nm}$, $\lambda_{em}=567\text{ nm}$)，可用来标记追踪体内外多种细胞。在细胞毒性分析，荧光蛋白、抗体或 DNA 染料在该区域发出的紫色、绿色、红色和远红外等，不会与 PKH26 产生干扰。PKH26 最常被用于基于染料稀释增殖的分析的染料稀释应用，包括建立抗原特异性前体频谱和正常或肿瘤组织中静止或缓慢

干细胞或祖细胞的鉴定。同时 PKH26 也可用于监测外来病毒、血小板和其他纳米颗粒的摄入；干细胞分裂过程中膜的分配；细胞-细胞之间膜的转移；细胞吞噬作用；抗原呈递；粘附；通过间隙连接的信号传递；以及组织切片中的神经元迁移。PKH26 荧光稳定性很强，特别是标记的细胞的研究周期超过一周时 PKH26 被用于体内细胞追踪研究。

试剂盒组份:

产品编号	GPKH26 染料	稀释液 C	价格
GPKH26-1	0.1mL	10mL	900 元

*MINI26 试剂盒推荐用于小的或初步试验研究，PKH26GL 用于体内实验研究

储存条件:

避光冷藏，使用前检查结晶，如有结晶需在 37°C 水浴溶晶。因在乙醇中贮藏，所以要盖紧防挥发。稀释液 C：常温或冰箱保存，无防腐剂和抗生素，保持无菌。

注意事项:

- 染料的工作液随用随配，不要将配好的染料贮存，影响染色效果。
- PKH26 染色过程中，不能存在叠氮化物或代谢毒性物。
- 虽然贴壁细胞也可以染色，但为获得均匀染色，单细胞悬液最佳。因此用蛋白酶 (trypsin/EDTA) 将贴壁细胞消化成单个悬浮细胞后再染色效果更佳。
- 染色前去除血清和脂质以提高染色效果。
- 盐的存在会导致染料形成颗粒，干扰染色反应。因此，加染料前细胞重新悬置是很重要的。染料应直接加到细胞悬液中，而不是加到细胞团上。
- 过度的细胞标记将导致膜完整性丧失、细胞数量下降。本样品细胞和染料浓度是参照浓度适合大部分细胞，但最

佳染料/细胞由使用者根据细胞类型和实验目的决定，另外使用者还要评估细胞的生存力（排碘），荧光强度，荧光峰值的变异系数，染色的均匀性。

● 染色浓度根据细胞种类的不同和每孔内细胞数量的多少而异。不同的细胞种类标记后可以示踪的代次或时间差异较大，请根据实际情况或参考文献进行检测。

操作注意：快速均匀的混合对标记也很重要，为获得最佳效果应采取下述措施：1)

混合时细胞悬液和工作染液应等量；

2) 避免染太多(>5ml)或太少 (<100ul) 的液体。避免用沾血清的移液管加染料；

3) 液体量应尽可能精确以保证细胞和染料浓度被精确复制。

4) 染料和稀释液作用于细胞的时间尽量短，有一定毒性。可用稀释液按上述步骤作用于细胞看其受损程度。

5) 加等量血清终止反应，在终止染色反应前别离心稀释液 C 中的细胞，清洗时用含血清培养基可增加清洗效果。

6) 细胞应单独移至新试管中离心，清洗 3 次时不用稀释液 C 而用培养基。

7) 可用于体外干细胞、淋巴细胞、单核细胞、内皮细胞等的标记较理想。

8) 全细胞标记应先于单抗染色的标记，当 4°C 单抗染色时细胞跟踪探针将保持稳定；如后于单抗染色的标记，很可能会出现“盖帽”现象。

9) 染色细胞用 2% 多聚甲醛固定稳定性达 3w 以上。

所需材料：

均匀的单细胞悬液；含血清培养基；无血清培养基或不含 $\text{Ca}^{2+}\text{Mg}^{2+}$ 的 PBS 液；血清、白蛋白或与培养基兼容的蛋白源；聚丙烯锥形离心管；温度可控的离心机 (0-1000g)；荧光分析仪 (荧光计，荧光显微镜，流式细胞仪，荧光图象分析仪)；超净台；细胞计数仪；载玻片。

操作步骤：

1. 一般细胞膜标记

亲脂性染料结合到细胞膜上完成标记。染色强度是染料浓度和细胞浓度的函数，与渗透性无关。因此，保证染料添加量不过量非常关键。过标记的细胞将会导致细胞膜完整性缺失和或降低细胞活性。

下列过程可用于体内体外细胞的标记，包括干细胞、淋巴细胞、单核细胞、内皮细胞、神经细胞或者任何其他细胞。体内细胞的标记过程需一定的改进，如血小板的染色，或者选择性标记吞噬细胞。

下述染色过程中细胞浓度和染料浓度代表操作的起始浓度。该浓度被证明适用于多种细胞。使用者需通过评估染色后细胞活率（如，PI 染色）、荧光强度、染色均匀度及是否对所研究细胞功能有影响等，根据实验目的，确定最优的染料浓度和细胞浓度。

无菌操作示范步骤 (总体积 2ml, 染色终浓度为 2×10^{-6} M PKH26 染料和 1×10^7 细胞/ml, 所有操作在 20~25°C)

1. 胰酶和/或 EDTA 消化细胞形成单细胞悬液，将 2×10^7 个细胞于锥形离心管中，用无血清培养基洗一次。

注意：血清蛋白和脂质会与染料结合，降低与细胞膜结合的染料浓度。最好在用稀释液 C 重悬细胞染色前（第四步）用无血清培养基或缓冲液洗细胞一次（第一步）。

2. $400 \times g$ 离心 5 分钟形成松散的细胞团。

注意：PKH26 染料不能直接加到离心沉淀中，这样会造成细胞染色不均一和细胞活力降低。

3. 离心后，小心吸弃上层清液，细胞团上剩余液体 < 25ul。

注意：为得到可重复的实验结果，在用稀释液 C 重悬时，减少残留培养基或缓冲液体积非常重要。

4. 加入 1mL 稀释液 C，用移液管轻轻吹打混匀，制备 2× 细胞悬液。重悬细胞保证完全离散，别震荡，不要让细胞在稀释液 C 中保存太长时间。

注意：生理盐的存在会使得染料结团并大幅降低染色效率。需确保染色时细胞悬浮于稀释液 C 中，不含培养基或缓冲盐。

5. 临染色之前，将 4μL PKH26 乙醇溶液加入 1mL 稀释液 C 中，充分混匀，配制的 2× 染色液 ($4 \times 10^{-6} M$)。

注意 1：为减少乙醇对细胞活率的影响，步骤 5 加入的染料使得步骤 6 中乙醇最终浓度不能超过 1-2%。

注意 2：如果所需染料最终浓度 $< 2 \times 10^{-6} M$ ，需用 100% 乙醇将 PKH26 稀释于另一单独的容器中，以确保实验结果的可重复性。

6. 快速将 1mL 2× 细胞悬液 (步骤 4) 加入 1mL 2× 染色液 (步骤 5) 中，立即用吸管均匀快速混合样品，因为均匀的染色是在瞬间发生的。(最终细胞浓度为 $1 \times 10^7 / mL$ ，PKH26 浓度为 $2 \times 10^{-6} M$)。

注意：由于染色瞬间完成，快速将细胞与染料混匀对得到明亮、均匀和可重复的标记结果非常重要。为获得最佳效果应采取下述措施：

- 不要将 PKH26 染料直接加入含 2× 细胞的稀释液 C 中。
- 将 2× 细胞悬液 (步骤 4) 与 2× 染色液 (步骤 5) 等体积混合。
- 调整 2× 细胞和 2× 染料的浓度避免染色体积太小 ($< 100 \mu L$) 或太大 ($> 5 mL$)。
- 用电动移液器快速将细胞和染料混匀。血清移液管混匀速度太慢而使得染色不均匀。震荡和旋涡震荡混匀同样混匀较慢，染色均一性较差。
- 分配体积尽量准确，以保证样品与样品之间，实验与实验之间的可重复性。

7. 混匀后的染色的细胞 25°C 孵育的 2-5min，定时轻轻颠倒离心管保证在 25°C 充分混匀。由于染色速度较快，延长孵育时间对实验没有帮助。

注意：让细胞在染色液中停留尽量短的时间，同时保证得到理想的染色强度。因为稀释液 C 缺少生理性盐，过长时间的暴露在稀释液 C 中会造成某些细胞活力降低。如果不能确定其影响程度，可增加仅作稀释的对照组和只用乙醇而不用染料染色的对照组。

8. 加入等体积的血清 (2mL) 或加入等量血清或 1% BSA 中止染色反应，孵育 1min 以结合多余的染料。

注意 1：血清 (或等效的蛋白浓度) 为最优的终止液。如果用完全培养基 (含血清的组织培养基) 替代，添加体积为 10mL。

注意 2：不要通过加稀释液 C 或离心来终止反应。

注意 3：不要用无血清培养基或缓冲盐，他们会使染料产生聚集。染料聚集使清洗过程中无法洗净，使得分析过程中仍存在未标记的细胞。

9. 将细胞在 20-25°C 条件下 $400 \times g$ 离心 10 min，小心吸弃上清。用 10mL 完全培养基 (含血清的组织培养基) 重悬，将重悬液转移至另一新的无菌离心管中，20-25°C 条件下 $400 \times g$ 离心 5 min。用 10mL 完全培养基再清洗两遍以除去没结合的染料。

注意 1：将重悬液转移至新离心管中，减少了离心管壁残留的染料对洗涤效率的影响；注意 2：不要用稀释液 C 清洗。

10. 最后一步清洗后，用 10mL 完全培养基重悬细胞评估细胞回收率，细胞活率和荧光强度。离心重悬细胞至所需活细胞浓度。

注意 1：染色后的细胞可以用中性甲醛固定，避光条件下，荧光强度至少 3 周内保持稳定。

注意 2：染色荧光强度一般为背景的 100-1000 倍。虽然染色的 CV 值与细胞种类有关，但荧光分布应该尽量均匀对称。

11. 荧光显微镜/流式细胞仪分析细胞。检查细胞复苏情况、传代情况及荧光浓度。染色应均匀，比本底荧光高 100-1000 倍。

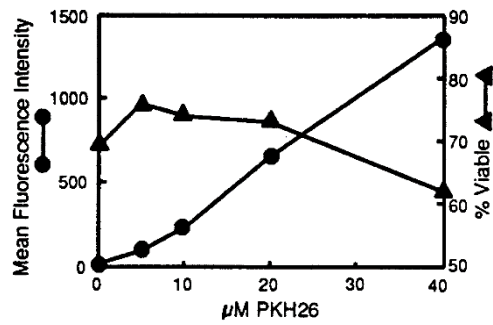


图 2. PKH26 染色优化

MC-38TIL 细胞用上述指定 PKH26 浓度染色，最终细胞浓度为 1×10^7 /ml。活力 (▲) 由 FITC 染色测定，平均荧光强度 (●) 用流式细胞仪检测。用 20 μM PKH26 标记后，抗肿瘤 TIL 特异性和效能没有改变。

2. 组织学:

制备和保存含 PKH 标记细胞切片时要冰冻切片和特殊的封固标本技术。

切片制备：

- 1、 切除组织后立即放入干冰冰冻。
- 2、 切片前 -70°C 保存。
- 3、 用 OCT (Tissue-Tek; Miles, INC.) 复合物制作冰冻切片。
- 4、 4-5um 组织切片。
- 5、 载玻片室温下干燥 1h。
- 6、 封固盖玻片用 1-2 滴氨基丙酸盐粘合剂酯胶。
- 7、 检查和拍片时用标准的滤光器如 FITC(PKH2 和 PKH67)或 TRITC (PKH26)。

复染切片：

- 1、 将玻片浸在丙酮液 24-48h，去除盖片；
- 2、 蒸馏水清洗去丙酮；
- 3、 复染切片易用 Mayer 或 Harris 苏木精；
- 4、 封固载玻片用 AS/AP 永久性水合封固液 (Bio/Can America, Inc. · Porland, ME) 注意：由于有机溶剂可析取 PKH 染液，而复染又吸收荧光，因而同时观察组织学和 PKH 荧光不可取。

PKH26 Red Fluorescent Cell Linker Kit

Product Description

The PKH26 Fluorescent Cell Linker Kits use proprietary membrane labeling technology to stably incorporate a yellow-orange fluorescent dye with long aliphatic tails (PKH26) into lipid regions of the cell membrane.^{1,2} The labeling vehicle provided in the kits (Diluent C) is an aqueous solution designed to maintain cell viability, while maximizing dye solubility and staining efficiency during the labeling step. Diluent C is iso-osmotic for mammalian cells and contains no detergents or organic solvents, but also lacks physiologic salts and buffers. The appearance of labeled cells may vary from bright and uniform to punctate or patchy, depending on the cell type being labeled and the extent to which membrane internalization occurs after labeling. However, PKH26 fluorescence is independent of pH within physiologic ranges and fluorescence intensity per cell is typically unaffected by the pattern of dye localization.

PKH26 fluoresces in the yellow-orange region of the spectrum (see Figure 1) and has been found to be useful for in vitro and in vivo cell tracking applications in a wide variety of systems. PKH26 is compatible with violet, green, red, or far-red viability probes used in cytotoxicity assays and with fluorescent proteins, antibodies, or DNA dyes emitting in these spectral regions. One of the most common uses of PKH26 is proliferation analysis based on dye dilution. Applications of dye dilution proliferation analysis include estimation of antigen-specific precursor frequencies and identification of quiescent or slowly dividing stem or progenitor cells in normal and neoplastic tissues.¹⁶⁻²⁰ PKH26 has also proven useful for monitoring: uptake of exosomes, viruses, platelets, and other nanoparticles; apportionment of membrane in stem cell division; cell-cell membrane transfer ; phagocytosis; antigen presentation; adhesion; signaling through gap junctions, and neuronal migration in tissue slices. Due to its extremely stable fluorescence, PKH26 is the cell linker dye of choice for in vivo cell tracking studies, particularly when labeled cells are to be followed for periods longer than a few weeks.

Components

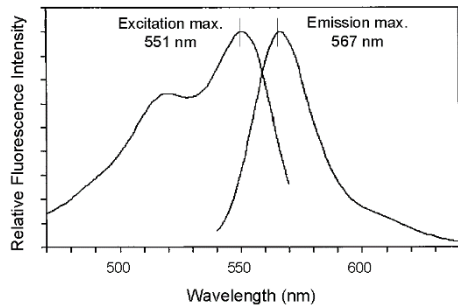
产品编号	GPKH26 染料	稀释液 C	价格
GPKH26-1	0.1mL	10mL	900 元

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Storage/Stability

The PKH26 ethanolic dye solution may be stored at room temperature or refrigerated. To prevent increases in dye concentration due to evaporation, keep the ethanolic dye solution tightly capped except when in immediate use. The dye solution must be protected from bright direct light and examined for crystals prior to use. If crystals are noted in the dye solution, warm slightly in a 37 °C water bath, and sonicate or vortex until

redissolved. Diluent C may be stored at room temperature or refrigerated. If refrigerated, bring to room temperature before preparing cell and dye suspensions for labeling. Diluent C is provided as a sterile solution. Because it does not contain any preservatives or antibiotics, it should be kept sterile. Do not store dye in Diluent C. Working solutions of dye in Diluent C should be made immediately prior to use.



Procedures

A. General Cell Membrane Labeling.

Labeling occurs by partitioning of the lipophilic dye into cell membranes. Labeling intensity is a function of both dye concentration and cell concentration and is not saturable. Therefore, it is essential that the amount of dye available for incorporation be limited. Over-labeling of cells will result in loss of membrane integrity and reduced cell recovery.

The following labeling procedure can be used for in vitro or ex vivo labeling of stem cells, lymphocytes, monocytes, endothelial cells, neurons, or any other cell type where partitioning of dye into lipid regions of the cell membrane is desired. Modified procedures may be required for in vivo labeling, for labeling platelets, or for selective labeling of phagocytes in the presence of non-phagocytes.

General cell membrane labeling should be performed prior to monoclonal antibody staining. The membrane dyes will remain stable during the monoclonal staining at 4 °C; however, capping of the monoclonal antibodies is highly probable if the general cell membrane labeling is carried out at ambient temperature subsequent to antibody labeling.

The cell and dye concentrations given in the following procedure represent starting concentrations that have been found broadly applicable to a variety of cell types.⁷ Users must determine the optimal dye and cell concentrations for their cell type(s) and experimental purposes by evaluating post-staining cell viability (e.g., propidium iodide exclusion), fluorescence intensity, staining homogeneity, and lack of effect on cell function(s) of interest.

Note: No azide or metabolic poisons should be present at the time of staining with PKH26.

Note: Although adherent cells may be labeled while attached to a substrate, more homogeneous staining is obtained using single cell suspensions. Best results will be obtained if adherent or bound cells are dispersed

into a single cell suspension using proteolytic enzymes, e.g., trypsin/EDTA, prior to staining.

The following procedure uses a 2 mL of final staining volume containing final concentrations of 2×10^{-6} M of PKH26 and 1×10^7 cells/mL.

Perform all further steps at ambient temperature (20–25 °C)

1. Place a suspension containing 2×10^7 single cells in a conical bottom polypropylene tube and wash once using medium without serum.

Note: Serum proteins and lipids also bind the dye, reducing the effective concentration available for membrane labeling. Best results are obtained by washing once with serum-free medium or buffer (step 1) prior to resuspension in Diluent C for labeling (step 4).

2. Centrifuge the cells (400 x g) for 5 minutes into a loose pellet.

Note: The PKH26 ethanolic dye solution should not be added directly to the cell pellet. This will result in heterogeneous staining and reduced cell viability.

3. After centrifuging cells, carefully aspirate the supernatant, being careful not to remove any cells but leaving no more than 25 μ L of supernatant.

Note: For reproducible results, it is important to minimize the amount of residual medium or buffer present when cells are resuspended in Diluent C.

4. Prepare a 2X Cell Suspension by adding 1 mL of Diluent C to the cell pellet and resuspend with gentle pipetting to ensure complete dispersion. Do not vortex and do not let cells stand in Diluent C for long periods of time.

Note: The presence of physiologic salts causes the dye to form micelles and substantially reduces staining efficiency. Therefore, it is important that the cells be suspended in Diluent C at the time dye is added, not in medium or buffered salt solutions.

5. Immediately prior to staining, prepare a 2x Dye Solution (4×10^{-6} M) in Diluent C by adding 4 μ L of the PKH26 ethanolic dye solution to 1 mL of Diluent C in a polypropylene centrifuge tube and mix well to disperse.

Note: To minimize ethanol effects on cell viability, the volume of dye added in step 5 should result in no more than 1–2% ethanol at the end of step 6.

Note: If a final dye concentration $< 2 \times 10^{-6}$ M is desired, the most reproducible results will be obtained by diluting the PKH26 ethanolic dye solution provided in the kit with undenatured 100% ethanol to make an intermediate dye stock.

6. Rapidly add the 1 mL of 2x Cell Suspension (step 4) to 1 mL of 2 x Dye Solution (step 5) and immediately mix the sample by pipetting. Final concentrations after mixing the indicated volumes will be 1×10^7 cells/mL and 2×10^{-6} M PKH26.

Note: Because staining is nearly instantaneous, rapid and homogeneous dispersion of cells in dye solution is essential for bright, uniform, and reproducible labeling. The following measures have been found to aid in optimizing results:

- a. Do not add ethanolic PKH26 dye directly to the 2x Cell Suspension in Diluent C.
- b. Mix equal volumes of 2x Cell Suspension (step 4) and 2 x Dye Solution (step 5).
- c. Adjust 2 x cell and 2 x dye concentrations to avoid staining in very small (<100 μ L) or very large (>5 mL) volumes.
- d. Use a Pipetman or equivalent for rapid addition of cells and mixing with dye. Serological pipettes are slower and give less uniform staining. Mixing by “racking” or vortexing is also slower and gives less uniform staining.
- e. Dispense volumes as precisely as possible in order to accurately reproduce both cell and dye concentrations from sample to sample and study to study.

7. Incubate the cell/dye suspension from step 6 for 1~5 minutes with periodic mixing. Because staining is so rapid, longer times provide no advantage.

Note: Expose cells to dye solution and Diluent C for the minimum time needed to achieve the desired staining intensity. Since Diluent C lacks physiologic salts, longer exposure may cause reduced viability in some cell types. If such effects are suspected, include a diluent-only control and a mock-stained control using ethanol rather than dye.

8. Stop the staining by adding an equal volume (2 mL) of serum or other suitable protein solution (e.g., 1% BSA) and incubate for 1 minute to allow binding of excess dye.

Note: Serum (or an equivalent protein concentration) is preferred as the stop solution. Increase volume to 10 mL if complete medium is used instead of serum.

Note: Do not stop by adding Diluent C or centrifuge the cells in Diluent C before stopping the staining reaction.

Note: Do not use serum-free medium or buffered salt solutions, which cause formation of cell-associated dye aggregates. Dye aggregates act as slow-release reservoirs of unbound dye that are not efficiently removed by washing and can transfer to unlabeled cells present in an assay.

9. Centrifuge the cells at 400 x g for 10 minutes at 20~25 °C and carefully remove the supernatant, being sure not to remove cells. Resuspend cell pellet in 10 mL of complete medium, transfer to a fresh sterile conical poly-propylene tube, centrifuge at 400x g for 5 minutes at 20~25 °C, and wash the cell pellet 2 more times with 10 mL of complete medium to ensure removal of unbound dye.

Note: Transfer to a fresh tube increases washing efficiency by minimizing carryover of residual dye bound to tube walls.

Note: Do not use Diluent C for washing steps.

10. After the final wash, resuspend the cell pellet in 10 mL of complete medium for assessment of cell recovery, cell viability, and fluorescence intensity. Centrifuge and resuspend to desired final concentration of viable cells.

Note: Stained cells may be fixed with 1–2% neutral buffered formaldehyde and intensities are stable for at least 3 weeks if samples are protected from light.

Note: Staining is typically at least 100 – 1,000 times brighter than background autofluorescence. Intensity distributions should be symmetrical and as homogeneous as possible, although staining CV will depend on the cell type being stained.

B. Histology

Using DAB photo-oxidation and transmission electron microscopy it is possible to visualize internalization of PKH26 labeled membranes or nanoparticles at the ultrastructural level. PKH26 labeled adipocytes have been successfully identified in tissues subjected to standard paraffin embedding and sectioning, but such methods risk loss of intensity because they use clearing agents that may partially extract membrane lipids and lipophilic dyes.

Histologic studies of tissues containing cells labeled with lipophilic membrane dyes have typically been carried out on serial frozen sections or sections prepared after fixation in neutral buffered formalin prepared from 4% paraformaldehyde. These methods avoid quenching of fluorescence by absorbing dyes found in histologic counterstains. For studies where imaging is to be done on a single section, fluorescence microscopy should precede counterstaining.

The following methods were developed at the Pittsburgh Cancer Institute by Drs. Per Basse and Ronald H. Goldfarb for use with PKH26.

Fluorescence imaging of frozen sections:

1. Excise tissues to be sectioned and freeze immediately on dry ice.
2. Store tissues at -70°C prior to sectioning.
3. Mount frozen tissues using O.C.T. compound (Tissue-Tek; Miles, Inc.) or equivalent.
4. Prepare tissue sections.
5. Air dry slides for at least 1 hour at room temperature.
6. Mount coverslip using 1–2 drops of cyanoacrylate ester glue.
7. Examine or photograph sections using an appropriate filter setup (e.g., PE for PKH26).

Counterstaining of frozen sections:

1. Remove coverslips by soaking slides in acetone for 24–48 hours.
2. Rinse slides in distilled water to remove acetone.

3. Counterstain sections using stain of choice. Satisfactory results have been obtained using Mayer's or Harris hematoxylin.
4. Mount slides using AS/AP permanent aqueous mounting medium (Bio/Can America, Inc., Portland, ME) or equivalent.

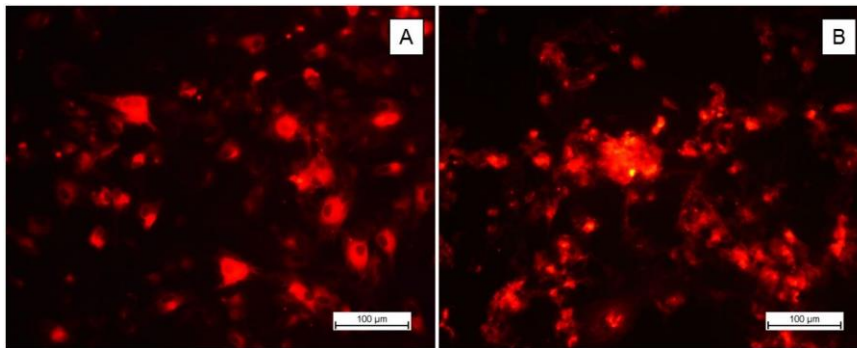
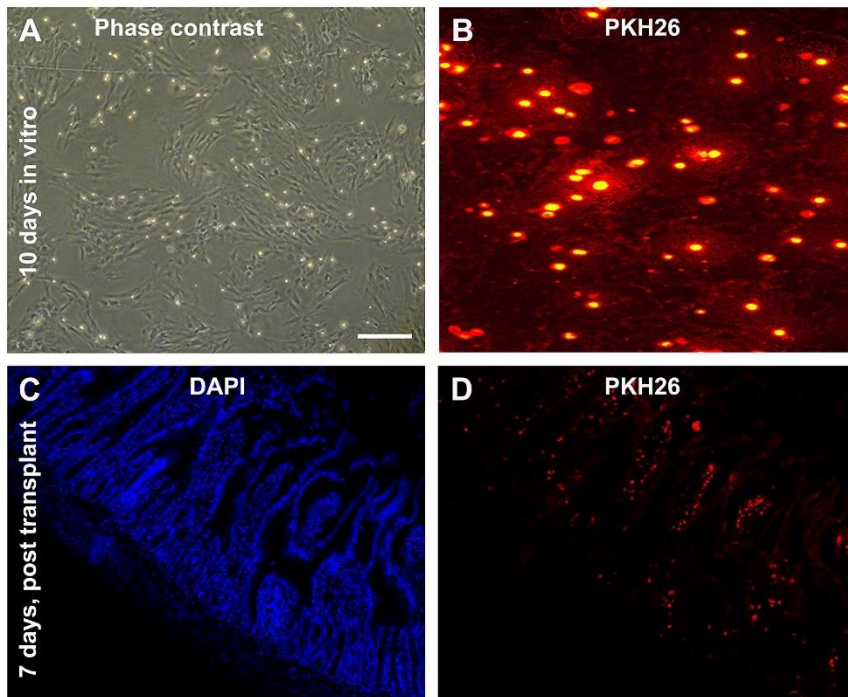


Figure Confirmation of cell labelling with the fluorescent dye, PKH26. (A) Osteoblastic cells grown on glass coverslips after 24 h; (B) osteoblast cell culture after 14 days



(A) A large number of cells adhering to a culture dish with spindle-shape morphology at 10 days in vitro. (B) Culture cells pre-labeled by PKH26 show bright red fluorescence. Panels (C) and (D) show the distribution of PKH26 positive BMMSCs in the wall of the small intestine 7 days after infusion, with the cells mostly localized to the mucosal region. Scale bar = 500 µm in (A) applying for (C, D), equivalent to 125 µm for (B).

