A Low-Cost Technique for Intraoperative Imaging of Cell Delivery and Retention in a Model of Delayed Wound Healing

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Objective: Techniques to validate successful delivery of cell products are expensive, time-consuming, and require transport of the animal to imaging facilities, preventing their widespread use as documentation tools. The goal of this study was to determine if a low-cost portable microscope could provide sufficient performance to be used to document delivery of cell products and track retention over time.

Approach: A Dino-Lite fluorescence microscope and an Odyssey CLx whole-animal scanner were compared on the basis of resolution, sensitivity, and linearity. The impact of different injection profiles on image quality was also compared and the system was used to track cells, injected freely or on scaffolds, in a model of diabetic wound healing.

Results: Both systems were able to detect 50 fluorescently labeled cells and there was a linear relationship between the fluorescence signal and cell number *in vitro*. *In vivo*, both systems were found to be nonlinear, but highly correlated with one another. The Dino-Lite system was able to distinguish between depth of injection, diffuse injections, subcutaneous injections, and failed injections.

Innovation: In contrast to traditional imaging systems, the technique presented here is affordable, rapid enough that it can be used to validate every injection, and can be brought to the animal, reducing handling and stress that may interfere with wound healing processes.

Conclusion: Collectively, we found that the speed, affordability, and portability of handheld microscopes combined with their technical capabilities make them a valuable and accessible tool for routine validation, documentation, and tracking of cell products delivered to wounds.

Keywords: MSC, mesenchymal stem cell, diabetic wound healing, handheld microscope, cell delivery, TallyHo mice

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INTRODUCTION

WHEN REFINING WOUND healing therapies that incorporate living cellular products, ensuring consistent delivery and retention of the product in the wound site is imperative. If an animal fails to respond to a therapy, researchers need to be able to determine the cause of the failure: insuffi-

cient dosing, poor proximity to the wound margins, lack of persistence, or change in cell phenotype. Thus, there is a need for techniques to validate the initial dose and location of cells at the time of administration, either alone or on scaffolds, and to track the persistence of cells in the wound throughout the healing

process. While multiple technologies are currently available to track cells *in vivo*, most require the animal to be taken to an imaging suite, are time-consuming, and remain prohibitively expensive. Herein, we present a protocol that utilizes a low-cost, handheld fluorescence microscope to rapidly validate and record cell transplantation within an operative environment and demonstrate its utility in monitoring the *in vivo* location and persistence of cells transplanted into a TallyHo excisional wound model. The technical capability of the system combined with its logistical advantages makes it attractive for routine validation and tracking of cell transplantation into the wound environment.

CLINICAL PROBLEM ADDRESSED

Diabetic wounds are the leading cause of non-traumatic lower extremity amputations¹ and are estimated to afflict 25% of diabetic patients over the course of their lifetime.² Due to the large number of patients and shortcomings of current therapies, there is a tremendous need for more efficacious therapies. The complexity of the disease environment and the severity of dysfunction of resident cells make tissue engineering and regenerative medicine solutions that utilize healthy living cells attractive candidates for new therapies. However, preclinical animal testing of these living therapeutics is limited due to different responses to therapy between animals, inconsistency in therapy application, and animal manipulation of the therapeutic. Therefore, it is imperative to document delivery of cell products, improving the relevance of data collected from preclinical models and the assessment of novel cell therapies. Unfortunately, cell-tracking techniques used today are expensive, time-consuming, and require transport of the animal to imaging facilities, preventing their widespread use as documentation tools.

MATERIALS AND METHODS

Cell culture

Human mesenchymal stem cells also known as mesenchymal stromal cells or MSCs were obtained from RoosterBio and cultured in minimal essential medium alpha (MEM- α) media supplemented with 15% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. MSCs from RoosterBio are evaluated according to the ISCT minimal criteria³ and are specifically >95% positive for CD73a, CD90, and CD105, <2% positive for CD11, CD14, CD19, CD34, CD45, CD79a, and HLA-II, and capable of multilineage differentiation. Cell cultures were seeded at 3,000 cells/cm² and passaged when

plates reached 70% confluence for all experiments. All cells used in this study were at passages 3–6 at the time of use.

Adenoviral amplification

Adenovirus expressing the mCherry fluorescent protein was procured from the Viral Vector Core at the University of Iowa (Ad5CMVmCherry, Cat No. VVC-U of Iowa-537). HEK293A cells grown to 90% confluence in a 150-mm Petri dish were infected with 40 μ L of Ad5CMVmCherry (7×10^{10} pfu/mL). After cell detachment from the plate and confirmation of cell expression of mCherry, cells were collected and then lysed through freeze/thaw cycling. The media were spun at 800 g for 5 min to pellet cell debris. Supernatant was transferred and stored at -80°C (generation 1 virus). HEK293A cells were grown to 90% confluence and infected with 80 μ L of generation 1 virus. After confirmation of mCherry expression and detachment from the plate, the virus was collected and frozen for labeling of MSCs as before (generation 2 virus). Infectious unit (IFU) of the adenovirus was calculated by infecting 500,000 HEK293 cells in a 12-well plate with 10 μ L, 1 μ L, 100 nL, 10 nL, and 1 nL of crude virus. After 48 h, the cells were visualized on a fluorescence microscope and cells were averaged from the condition that contained on average 10–50 cells expressing mCherry per $20 \times$ field of view (FOV). The IFU was calculated by averaging the number of mCherry⁺ HEK293 cells in 20 fields of view with IFU/mL = (average no. of mCherry⁺ cells $\times$ 597 fields of view per well)/(mL of virus added). In this case, the wells containing 10 nL of virus had an average of 12 mCherry⁺ cells in them. Therefore, IFU/mL = $(24 \times 597)/(1\text{E}-5 \text{ mL}) = 1.4\text{E}9 \text{ IFU/mL}$.

Cell staining and tracking

To be able to track MSCs both with our handheld fluorescence microscope and the Odyssey CLx whole-animal scanner, MSCs were colabeled with DiR and transduced to express mCherry. For adenoviral induction of mCherry expression, 250 μ L of generation 2 adenovirus at a stock concentration of $1.4\text{E}9 \text{ IFU/mL}$ yielding a multiplicity of infection of 440 IFU/cell encoding for mCherry was incubated in 4 mL of MEM- α supplemented with 25 mM CaCl₂ (Sigma) for 20 min at room temperature. MSCs were washed in phosphate-buffered saline (PBS) and then incubated with the virus solution at 37°C for 30 min. Cells were washed in complete MEM- α and allowed to recover for at least 4 h before replating. Cells were harvested 2 days after adenoviral transduction to allow for stable expression of mCherry and then stained with DiR immediately before plating in experimental conditions. MSCs

were lifted with accutase, washed in PBS, and incubated with DiR dye (Biotium) diluted to 0.1 mg/mL in media for 20 min at 37°C. Cells were then rinsed with PBS, pelleted, and resuspended in fresh media before use in either *in vitro* or *in vivo* assays. This dose of virus had been confirmed to maintain >95% MSC viability.

Mice and animal care

Animal experiments were approved by the University of Iowa IACUC committee. Eight-week-old male TallyHo mice were purchased from the Jackson Laboratories and housed for an additional 6 weeks on an NIH-31 modified open formula mouse diet (Envigo 7913). At 10–12 weeks, increase in urination became apparent, indicating induction of diabetes. At weeks 15, 16, and 17, blood glucose of each mouse was measured and recorded to confirm hyperglycemia, followed by wounding procedures, to enable *in vivo* tracking of cells administered to the wound bed. After wounding, animals were singly housed to prevent animals from interfering with each other's bandages. Since hyperglycemic phenotype penetrance is less than 100% in the TallyHo mice,^{4,5} the glucose levels of all were measured before surgery and postsurgery to ensure that blood glucose levels of animals used were >200 mg/dL. As the focus on this project was on developing the cell-tracking technique in a chronic wound model, only diabetic mice were used for all experiments.

Full-thickness excisional wounding and stenting

An adaptation of a previously published protocol was used to create two full excisional wounds on the backs of each mouse (Fig. 6A, B).⁶ Mice were anesthetized with isoflurane and maintained at a level of anesthesia to suppress pedal reflexes. Glucose was measured with a handheld meter to ensure all mice were diabetic (plasma glucose >200 mg/dL, average 485 ± 144 mg/dL). All mice were shaved and depilated by applying Veet hair removal cream for 5 min. The cream was removed thoroughly with 70% ethanol wipes. The site was then sterilized using three alternating applications of 70% ethanol and betadine solution before wounding. Mice were placed on their side, and a 5-mm biopsy punch was pushed through a dorsal skin fold to create a full-thickness excisional wound on each side of the midline near the scapula. To prevent the wound from closing through contraction, each wound was stented by suturing an autoclaved stainless steel locking washer (Item No. 19NP58; Grainger) with a 6.5 mm inner diameter around each wound using 5.0 silk sutures.

Intraoperative *in vitro* and *in vivo* validation of cell transplantation

For *in vitro* cell dosing, DiR/mCherry-labeled MSCs were plated in 20 μ L droplets on the bottom of a 150-mm Petri dish and allowed to attach overnight. Each 20 μ L droplet contained 250×10^3 , 150×10^3 , 75×10^3 , 50×10^3 , 25×10^3 , 10×10^3 , 1×10^3 , 500, 100, or 0 cells. Droplets were then imaged with a Dino-Lite Edge digital microscope with a filter set optimized to detect mCherry (AM4115T-RFYW) at a working distance (WD) of 20.6 mm (40 $\times$) with a 250 ms exposure time. The 250×10^3 , 1×10^3 , and 50 cell conditions were also imaged at a WD of 10.6 mm (60 $\times$) and 62.6 mm (20 $\times$). The plate was then re-imaged on an Odyssey CLx far-red imaging system (LI-COR) using 21 μ m spatial resolution, lowest image quality, L1 700 nm laser power, L2 800 nm laser power, and scanning offset of 0.0 mm. The Odyssey settings were determined in pilot studies used to identify settings that produced a focused quantifiable signal in a reasonable amount of time. The settings used allowed for detection of the highest dose of cells using our staining protocol without saturating the signal of any one pixel. Note that the quality parameter refers to the scan speed. Higher quality settings result in more data collected; however, they increase scan time dramatically (*i.e.*, >30 min/mouse). In a scenario where there would be movement of the animal (*e.g.*, breathing), the quality parameter would have to be set lower or movement artifacts would be picked up by the scanner.

For initial *in vivo* dosing experiments, TallyHo mice were injected with 250×10^3 , 75×10^3 , 25×10^3 , 1×10^3 , 500, and 0 MSCs stained with DiR and virally induced with mCherry dyes. Subcutaneous injections were performed on the dorsal side of the mouse to either side of the spine. Varying depth injections of 75×10^3 MSCs were performed at 0.5, 1.8, 3.1, 4.4, 5.7, and 7.0 mm into the dorsal side of the mouse. Depth of injection was controlled by limiting the length of the exposed needle using stiff silicone spacers. At all injections, moderate pressure was applied to the syringe to ensure that the syringe was at the measured depth. The length of the exposed needle was measured for each injection using digital calipers. Injection points were imaged with the Dino-Lite system at a WD of 20.6 mm (40 $\times$) and exposure of 0.25 s. Mice were then placed on the Odyssey scanner dorsal side down and imaged with 21 μ m spatial resolution; lowest image quality; 700 nm laser power of 1; 800 nm laser power of 3.5; and scanning offset of 4.0 mm.

MSC loading onto gelatin microcarriers

An adaptation to a gelatin microcarrier (MC) protocol was used to create a scaffold system for

MSCs.⁷ Briefly, gelatin MCs were prepared by crosslinking 5% w/v gelatin (Fisher Scientific) with 1.25% v/v glutaraldehyde (25% aqueous solution stock; Alfa Aesar) for 1 day. Residual glutaraldehyde and aldehyde groups were reduced using two treatments with 100 mM sodium borohydride (Thermo Fisher) for 24 h. After neutralization, the crosslinked gelatin changed from brown to a white color. Crosslinked gelatin was lyophilized, ground, and sieved to generate MCs 100–300 μm in diameter. The MCs were then rehydrated in media for 24 h to remove any residual sodium borohydride and allow adsorption of fibronectin onto the gelatin scaffold, rinsed, and then lyophilized. To sterilize the gelatin, 200 μL of 70% ethanol was added to each tube and ethanol was evaporated overnight under an ultraviolet (UV) sterilization light; 400×10^3 MSCs modified with mCherry were then seeded in 100 μL of media onto 15 mg of MCs. Cells were allowed to attach for 1 h, and then 1 mL of media was added incubated overnight.

Serial monitoring of cell retention

TallyHo mice wounded as described above and MSCs modified with mCherry were injected subcutaneously around the wound bed or topically applied at 200×10^3 cells per wound, 1 day after wounding. Free MSCs were harvested, washed in PBS, and resuspended at 4 million cells per milliliter in PBS. Free MSCs were loaded into insulin syringes and 50 μL of total cell suspension was injected in three to five areas around the perimeter of the excisional wound site. MSCs on MCs were applied directly to the wound bed to achieve delivery of 200×10^3 cells per wound. All wounds were bandaged after application of cells.

At days 3, 7, and 8 for the PBS and MSC-only groups and days 3 and 5 for the MC and MSC/MC groups, the mouse wound bandages were removed and fluorescence images were taken using the Dino-Lite camera in a dark room. Mice were anesthetized with isoflurane and positioned directly under the Dino-Lite system and imaged as described above.

RESULTS

Handheld microscope setup and configuration

The Dino-Lite handheld microscope (Fig. 1A) has the ability to acquire both bright-field and fluorescence images. The AM4115T-RFYW Dino-Lite system configuration is optimized for detection of the mCherry fluorescent protein; however, configurations can be customized to match the excitation and emission characteristics of a variety of fluorophores.

To begin, a basic understanding of the relationship between the FOV, WD from the microscope to the focal plane, and sensitivity was examined. FOV and WD from the sample are inversely proportional and both are directly tied to the level of magnification (Fig. 1A, B). Depending on the FOV requirements, images from 5.9×4.7 to 19.6×15.7 mm in size can be acquired with WDs ranging from 0.86 to 6.26 cm from the focal plane of the sample. The smaller the FOV, the greater the resolution and sensitivity of the image (Fig. 1C), therefore the cell detection limit will be higher for larger fields of view. Spatial resolution of the Dino-Lite system is dependent on magnification. At $20\times$ magnification, resolution is 15 μm and spatial resolution at a $40\times$ magnification is 7.6 μm . Thus, care must be taken in selecting the appropriate WD with the Dino-Lite system to capture the necessary image area.

Range and sensitivity of detection of fluorescent cells under ideal conditions

To characterize the linearity of the Dino-Lite microscope, we compared its range and sensitivity with a robust whole-animal imaging system, the Odyssey CLx infrared scanner. MSCs colabeled with mCherry (optimal spectral characteristics for the Dino-Lite system) and DiR (optimal spectral characteristics for the Odyssey scanner) were plated in 20 μL droplets at concentrations ranging from 50 to 250,000 cells per droplet and imaged with both imaging systems under preoptimized settings. Qualitatively, both systems were able to detect small clusters of cells down to the 50-cell condition (Fig. 2A), while quantitatively, based on mean fluorescence intensity (MFI), the Odyssey system had a lower limit of detection (50 cells) compared with background. Both the Odyssey and Dino-Lite systems produced calibration curves with a linear relationship between cell number and MFI (Fig. 2B–E). For optimal prediction of cell number, two distinct fit curves were produced, one for doses ranging from 0 to 1,000 cells and another for detection of 10,000–250,000 cells. Thus, under ideal acquisition settings in which the sample resides in a single focal plane, the Dino-Lite and Odyssey imaging systems performed similarly and both produced a quantitative assessment of cell number over a large range of cell doses.

In vivo relationship between cell number and fluorescence intensity

Due to absorption of light by tissues, as well as differences in the depth of injection and dispersion of cells within a tissue, *quantitative* tracking of cell numbers using light intensity-based modalities is challenging. We wanted to determine if the ability

Figure 1. Magnification can be set on the Dino-Lite system to optimize WD, FOV, and fluorescence intensity. Dino-Lite setup (**A**), where variation in magnification produces a nonlinear relationship with WD and a linear relationship with FOV (**B**). (**C**) Dino-Lite magnification modulates fluorescence intensity of cells. FOV, field of view; WD, working distance.

to focus the Dino-Lite system on a specific focal plane for each injection site would result in improved quantification of cell number *in vivo* compared with a traditional whole-body scanner. Thus, we subcutaneously injected defined numbers of labeled MSCs into cadaver mice and imaged with both the Dino-Lite and Odyssey systems. While DiR fluorescence emission had less interference from the tissue (Fig. 3A, B), both systems were able to detect cell numbers as low as 1,000 cells injected subcutaneously. As seen in Fig. 3, the Odyssey system can image the entire mouse in one scan, while the Dino-Lite system requires a separate image for each injection site. However, most wound healing studies are performed with wounds smaller than 10 mm in diameter, making the FOV of the Dino-Lite system adequate for use as a documentation camera for wound healing experiments.

Because each Dino-Lite image could be individually focused on the injection site, the variability between repeat measurements was lower for the Dino-Lite compared with the Odyssey system. In addition, the acquisition time for the Dino-Lite system took seconds, while the Odyssey scan took ~20 min per image. To adjust the focus of the Odyssey scanner, by changing the scanning offset parameter, an additional 20-min acquisition would have been required for each focal plane, making real-time focusing logistically impractical. For both the Dino-Lite and Odyssey systems, there was a similar nonlinear relationship between cell number and MFI. Additionally, a high degree of correlation between measurements obtained with the individual systems was achieved (Fig. 3C–E). Thus, the Dino-Lite system appears to perform equally to the Odyssey scanner with respect to

Figure 2. DiR/mCherry-stained MSCs display a dose-dependent fluorescence signal *in vitro* through both Odyssey and Dino-Lite imaging. **(A)** DiR/mCherry-stained MSCs in Petri dish imaged by the Odyssey fluorescence scanner and Dino-Lite system (40 $\times$ magnification). MFI of MSCs over dosing range ($n=3$) for the Odyssey fluorescence scanner **(B, C)** and Dino-Lite system **(D, E)**. Scale bar 3 mm. MFI, mean fluorescence intensity. MSC, mesenchymal stem cell or mesenchymal stromal cell.

detection limit as well as the ability to detect cells both *in vitro* and *in vivo*. While robust *in vivo* quantitative assessment is not possible with either system, a qualitative assessment can be obtained with both allowing for confirmation and qualification of each injection as was explored next.

Validating consistency in cell product delivery

To determine if the Dino-Lite system was able to detect changes in fluorescence intensity that arise due to inconsistencies in delivery technique, we took images of 150,000 cells injected at varying depths in TallyHo mice. As seen in Fig. 4, injection depth drastically changed the signal acquired by the Dino-Lite system. After deep injections, the Dino-Lite system detects a well-defined injection track. At medium injection depths, 1.8–4.4 mm, the fluorescence signal transitions from a single point

to a less defined point among diffuse fluorescence radiating from the injection track as the Dino-Lite system excites and identifies cells that are dispersed under the skin. Finally, at the shallowest depth, 0.5 mm, the majority of cells flowed out of the tissue, leaving a diffuse layer of cells on the surface of the skin. Thus, with the Dino-Lite system, we were able to distinguish successful point injections from failed injections in which the cell product flowed out of the injection site.

In addition to failed injections, we hypothesized that other manipulations of mice during handling or deviations in presurgical preparation could impact the signal detected by the handheld microscope. Thus, we staged common scenarios known to arise in wound studies, including application of pressure on the wound site, the presence of hair, and removal of the cell product by mechanical

Figure 3. Dino-Lite and Odyssey imaging quantification of MSC fluorescence shows a similar dose dependence *in vivo*. DiR/mCherry-stained MSCs subcutaneously injected into mouse skin imaged by the Odyssey scanner (A) and Dino-Lite system (B). Quantification of mean fluorescence signal of MSCs for the Odyssey scanner (C) and Dino-Lite system (D). Spearman correlation comparison of measured intensities from Dino-Lite and Odyssey imaging (E). Scale bar 3 mm.

abrasion (Fig. 5). While subcutaneous point injections in TallyHo mice have a bright injection point (Fig. 5A), application of pressure to the injection site, as might occur during bandaging, causes the injection point to become less distinct (Fig. 5C). Failure to completely depilate the surgical site before injection can also lead to variations in the fluorescence profile as hair can both absorb and

scatter the excitation and emission light traveling from the microscope and cells, respectively (Fig. 5D). Finally, topically applied cells, (whether intentionally or unintentionally) due to injection site squirt out, create a bright patterned texture (Fig. 5E). However, if cells are removed, to clean the surface after a failed injection or due to activity by the animal, residual cells can still be detected on

Figure 4. Injection depth impacts MSC fluorescence intensity and distribution of signal. Fluorescence signal from 150,000 mCherry MSCs as measured by the Dino-Lite system at 7.0-mm (A), 5.7-mm (B), 4.4-mm (C), 3.1-mm (D), 1.8-mm (E), and 0.5-mm (F) depths in mouse skin. Scale bar 3 mm.

Figure 5. Dino-Lite imaging to document and characterize quality of cell application *in vivo*. **(A)** Subcutaneous point injection of 150,000 mCherry MSCs. **(B)** Deep point injection 7 mm into mouse tissue. **(C)** Point injection of cells with subsequent mechanical stress applied to tissue causing cell dispersion. **(D)** Point injection where cell signal has been obscured by mouse hair. **(E)** Topical squirt out of cells onto mouse skin. **(F)** Verification of topical cell removal. Scale bar 3 mm.

the skin surface (Fig. 5F). While qualitative, the Dino-Lite images were able to validate initial injection success and track the relative decline in remaining cells if the majority of cells are removed by physical abrasion. Combined with the speed and ease of acquisition, these results make the system attractive as a routine documentation system for wound healing studies of cell-based therapies.

Serial imaging to track persistence of cell product

Validation of initial application of cells is useful, but continued tracking of cells over time is also important for verification of relative cell decline throughout the duration of the study, particularly for slow healing models of disease. We sought to determine if we could successfully track MSC decline in TallyHo mice after topical delivery on a gelatin scaffold or through subcutaneous injection in the margins of an excisional wound (Fig. 6A, B). Fifteen-week-old TallyHo mice, with advanced hyperglycemia and impaired wound healing ($78\% \pm 12\%$ remaining wound area at day 7 postwounding), were wounded with two 5-mm full excisional wounds and each wound was stented as shown in Fig. 6A. A subtherapeutic dose of MSCs was administered on a scaffold topically or without a scaffold through subcutaneous injection the day after wounding (day 1). As can be seen in Fig. 6, the PBS and scaffold-only groups had minimal background signal, while both MSC groups were bright 3 days after injection. In the free MSC groups, fluorescence signal declined over time (Fig. 6C), whereas MSC signal in the topical

scaffold administration remained relatively constant (Fig. 6D-i). However, over the course of study, several mice were able to remove much of the cell-laden scaffold and this was documented with the Dino-Lite fluorescence imaging system (Fig. 6D-ii). Thus, in addition to initial confirmation of cell delivery, the Dino-Lite system was useful in tracking the relative decline of cell numbers present within a particular wound site. As the *in vivo* imaging is qualitative, the assessment was most useful in comparing time points within the same animal, allowing for detection of cell product removal due to scratching from the mice.

DISCUSSION

In the current study, we have shown that when compared side-by-side with an Odyssey scanner, the handheld Dino-Lite microscope performed similarly while providing significant logistical advantages. Several manufacturers sell handheld fluorescence microscopes for less than \$1,000, making it an accessible technology for most laboratories. In addition, the small footprint of the microscope and ability to operate off a laptop allow the microscope to be used in the operating suite, eliminating transport of the animal to an imaging suite. This has significant advantages in reducing the stress experienced by research animals, which can have dramatic effects on the animal's health. This technique can readily be adapted to larger animal models where other imaging modalities may not be available due to scanner size constraints. Finally, while the maximum range of

Figure 6. Fluorescently labeled MSCs can be tracked multiple days *in vivo*. **(A)** TallyHo mice were wounded, injected with PBS, and wound tissue was harvested on days 7 and 14. **(B)** After creation of wounds, metal washers (6.5 mm ID) were sutured in place to prevent wound closure and provide an image alignment reference. **(C)** Representative fluorescence Dino-Lite images of mCherry-labeled MSCs (400,000 cells) over study for PBS and MSCs only at 3 and 7 days after wounding. **(D)** Validation of MSC scaffold scratch out detection; (i) indicates wounds without detected scratch out and (ii) indicates wounds with scratch out detected. Scale bar 3 mm. PBS, phosphate-buffered saline.

detection of handheld microscopes is not as large as whole-animal imagers such as IVIS or Odyssey, the acquisition time is significantly shorter as each wound can be imaged in less than a minute with relatively high resolution and sensitivity (Fig. 2). Therefore, bright-field and fluorescence images can be acquired on the operating table, allowing for immediate feedback on the quality of cell administration. As we have shown, both Dino-Lite and Odyssey systems have a linear relationship between fluorescence and number of cells under ideal conditions (Fig. 2) and both systems have a nonlinear relationship *in vivo* (Fig. 3C, D) due to tissue absorption and cell distribution. With this nonlinear monotonically increasing trend, we compared the correlation between the two curves and found that the spearman correlation and p -value were $r = 0.885$ and $p < 0.001$, respectively, indicating a high degree of similarity in dose response of both techniques. Table 1 compares and contrasts a handheld fluorescence microscope with alternate imaging modalities. Collectively, these data validate that the Dino-Lite system is able to detect fluorescently

labeled cells with similar sensitivity to the Odyssey system and with the additional logistical benefits outlined in Table 1 and, as discussed above, demonstrate the utility of the Dino-Lite system as a valuable tool for validation of cell delivery and tracking of cell retention in dermal wounds.

Table 1. Comparison of imaging modalities

	IVIS	Odyssey	Handheld Microscope
Resolution	20–50 μm	21–337 μm	4.6–15 μm
Field of view	3.9–25 cm^2	Up to 25 cm^2	Up to 1.95 $\times$ 1.56 cm
Setup time	5–10 min	5–10 min	1 min
Acquisition time	1–10 s	20–30 min/animal	0–1 s
Spectral flexibility	Bright field	2-Color fluorescence: 700–900 nm	Bright field
	Luminescence		Single fluorescent color
	Multicolor fluorescence		
Price range (depends on configuration)	>\$100,000	>\$10,000	<\$1,000
Portable	No	No	Yes

IVIS, *in vivo* imaging system.

Figure 7. Workflow to tailor cell labeling and handheld microscope parameters to study needs.

As handheld microscopes feature a single fixed filter set and exposure durations up to 1 s, advanced planning is required to maximize the performance of the system. Figure 7 outlines the process of designing a study using a handheld microscope and the discussion below is focused on highlighting options and considerations that will impact the performance of the technique. Parameters such as mouse strain, route of cell delivery, size of wounds, and study duration are dictated based on the hypothesis being tested and should be considered first. The skin color of the mouse and rate of hair growth, which is dependent on strain, will impact the degree of optical interference, while the duration of tracking needed and expected persistence of cells in the wound bed will influence the choice of labeling strategy. Wounds <6 mm in diameter can readily be imaged at magnifications of $<50\times$ (Fig. 1B), but if a lower magnification is used, the sensitivity of the system will drop, which may necessitate an increase in labeling intensity. In addition, as demonstrated above, the dose of cells (Fig. 2) and depth of injection (Fig. 4) also impact the sensitivity of the imaging system. Thus, in the planning stage, each of these parameters should be considered, and the impact of the therapeutic strategy on the ability to image the cells should be considered when

selecting the labeling technique to ensure that adequate sensitivity is achieved.

Many labeling strategies exist for *in vivo* cell tracking, but each has advantages and disadvantages that need to be considered in light of the overarching goals of the experiment. For cell tracking *in vivo*, far-red emitting dyes allow for minimal light scattering and absorption in the tissue, allowing for greater depths of penetration. These dyes mainly fall into three categories: chemical dyes, fluorescent proteins, and quantum dots (Table 2). Chemical dyes consist of membrane dyes (e.g., DiI, DiR, PKH26, and Claret) and cytosolic dyes (e.g., Cell-Trace) that have been validated for *in vivo* cell

Table 2. Selection of compatible fluorescent tracers (pros/cons)

Type of Tracer	Advantages	Disadvantages
Chemical dyes (e.g., DiI, DiR, PKH26, Claret)	Rapid, viral-free, easily titrate intensity.	Signal dilutes with cell division, potential for transfer to neighboring cells.
Fluorescent proteins (e.g., mCherry, mNeptune)	Degraded when cell dies.	Potentially interferes with biological processes.
Quantum dots	Multiple far-red emission spectra available. Rapid labeling.	Cytotoxic, signal decays in cellular environment.

detection.^{8,9} These dyes have the advantage of rapid labeling (<1 h), require no viral or transfection reagents, and dye concentrations can easily be adjusted to gain greater sensitivity. However, these dyes can transfer to other cells and be diluted during cell division, making them nonideal for systems where transplanted cells undergo proliferation or studies where dye transfer events may dominate and cause an overestimation of cell retention. Fluorescent proteins overcome this problem as only living cells with the vector are capable of producing the fluorescent protein and fluorescence transfer is therefore unlikely. The downside of fluorescent proteins is that they typically require several days to establish stable expression and many cell types are difficult to transfect, and in some instances, fluorescent proteins can have an effect on cell function and immune detection.¹⁰ Alternatively, quantum dots have been developed with UV excitation and emission spectra in the red-near infrared wavelengths. Quantum dots are brighter and more photostable than traditional fluorophores and can be functionalized to allow for rapid labeling of cells (<1 h). Quantum dots are, however, pH sensitive and contain heavy metals, which can cause toxicity issues if they degrade.¹¹ Since quantum dots are typically attached to the cell membrane or internalized into the cell, such as membrane and cytoplasmic dyes, they are diluted in proliferating cells and vulnerable to being transferred to neighboring cells. Regardless of the choice of label, labeling procedures should be tested before use to ensure that they do not negatively affect the function and life span of cells *in vivo* when compared with unlabeled cells.

After an appropriate fluorophore is chosen for the study objective and duration, an appropriate handheld microscope that matches the spectral characteristics of the fluorophore can be selected. The Dino-Lite system utilized in the current study has a narrow band-pass excitation filter optimized for mCherry. In situations where biomaterials are autofluorescent, custom filter sets with narrow bandwidths optimized for specific fluorophores can be used to minimize autofluorescence. With a dye and microscope selected, the staining procedure can be optimized to enable detection of a desirable range of cell numbers. Care should be taken during labeling optimization to evaluate both sensitivity of detection and the impact of labeling on cell viability and function. Cell staining can be adjusted to allow for more sensitive detection of cells in higher numbers; however, this optimization comes at the expense of a higher limit of detection.

Before data collection, the handheld microscope should be calibrated to ensure that the same magnification is used at each time point. Using a calibration grid to measure the number of pixels over a known distance or a reference object, such as a metal washer stent, works well to calibrate the microscope. For imaging of stented wounds, the stent helps to ensure that the region of interest is within the FOV and that the wound is directly facing the camera. During validation, both a cell number standard curve using the optimized labeling strategy and a series of reference images depicting a successful application of cells should be generated to judge whether future cell applications have been successful. As seen in Fig. 5, different profiles of cell distribution generate distinct patterns that can then be used to determine if a cell product has been administered as intended. During the study, not only can relative cell decline be measured by comparing images of the same injection site at different time points but it can also be determined if the animals disturb or remove part of the treatment (Fig. 6).

In this study, a handheld fluorescence microscope could detect cell numbers with similar sensitivity, detection limit, and linear response as a whole-body fluorescence scanner. The handheld microscope's additional logistical advantages, namely portability and speed, made it an accessible and valuable tool to validate and track cell product delivery to wounds. The handheld microscope was affordable, rapidly captured images, and could be brought directly to the surgical suite, minimizing transport of animals. Like the traditional whole-animal scanner, the handheld microscope quantified changes in cell number under ideal conditions while providing qualitative information *in vivo*. The handheld microscope distinguished between differences in depth of injection, cell number, cell squirt out, and removal of cells. With appropriate planning and preparation to identify fluorophores, staining conditions, and filter sets, the handheld microscope is a powerful technique that makes routine validation and documentation of cell product delivery to wounds practical and accessible to all laboratories.

INNOVATION

In contrast to traditional imaging systems, the technique presented here is affordable, portable, and fast. This makes it accessible for all laboratories, reduces animal handling, and is fast enough to be used to validate every injection. The innovation of this technique lies in its ease of use, accessibility, and speed that make it both technically and

KEY FINDINGS

- The Dino-Lite system had a linear response to fluorescently labeled cell number similar to that of the Odyssey system.
- Depth of injection of cell products produced distinct fluorescence patterns.
- The handheld microscope could detect different types of injections, including point injections, topical cell application, and dispersion of cells over time from a point injection.
- Fluorescence microscope imaging could detect cell decline and scratch out of cells in a model of diabetic wound healing.

logistically valuable as a routine documentation system for cell therapy. This is a significant advancement that enables careful routine documentation of every cell injection in preclinical models, leading to significantly increased rigor in the development of cell-based wound therapies.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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Abbreviations and Acronyms

CaCl₂ = calcium chloride
 DiR = 1,1'-dioctadecyl-3,3',3'-
 tetramethylindotricarbocyanine
 iodide
 FOV = field of view
 IACUC = Institutional Animal Care and Use
 Committee
 IVIS = *in vivo* imaging system
 MC = microcarrier
 MEM-α = minimal essential medium alpha
 IFU = infectious unit
 MFI = mean fluorescence intensity
 MSC = mesenchymal stem cell or
 mesenchymal stromal cell
 PBS = phosphate-buffered saline
 UV = ultraviolet
 WD = working distance