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Dear Dr. Nandita Singh,

We hereby submit our manuscript entitled "**Real Time Video Projection in an MRI for the Characterization of the Neural Correlates Associated with Mirror Therapy treatment for Phantom Limb Pain"** which we have the pleasure to submit for publication in the Journal of Visualized experiments.

This manuscript has not been published or currently submitted for publication elsewhere. I certify that all authors have participated sufficiently in the writing of this manuscript, to take public responsibility for it. All authors approved the final version of this manuscript.

This manuscript describes a new approach combining neuroimaging and behavioral tasks in a real time video projection aiming to better understand the associations between mirror therapy treatments for phantom limb pain.

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TITLE:

Real-time Video Projection in an MRI for Characterization of Neural Correlates Associated with Mirror Therapy for Phantom Limb Pain

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KEYWORDS:

Phantom limb pain; fMRI, brain imaging; mirror therapy; amputation; neuroplasticity; sensorimotor cortex

SUMMARY:

We present a novel combined behavioral and neuroimaging protocol employing real-time video projection for the purpose of characterizing the neural correlates associated with mirror therapy within the magnetic resonance imaging scanner environment in leg amputee subjects with phantom limb pain.

ABSTRACT:

Mirror therapy (MT) has been proposed as an effective rehabilitative strategy to alleviate pain symptoms in amputees with phantom limb pain (PLP). However, establishing the neural correlates associated with MT therapy have been challenging given that it is difficult to administer the therapy effectively within a magnetic resonance imaging (MRI) scanner environment. To characterize the functional organization of cortical regions associated with this

rehabilitative strategy, we have developed a combined behavioral and functional neuroimaging protocol that can be applied in participants with a leg amputation. This novel approach allows participants to undergo MT within the MRI scanner environment by viewing real-time video images captured by a camera. The images are viewed by the participant through a system of mirrors and a monitor that the participant views while lying on the scanner bed. In this manner, functional changes in cortical areas of interest (e.g., sensorimotor cortex) can be characterized in response to the direct application of MT.

INTRODUCTION:

PLP refers to the sensation of pain perceived within the area corresponding to the missing limb postamputation[1,2]. This condition is a significant chronic health care burden and can have a dramatic impact on an individual's quality of life[3,4]. It has been suggested that alterations in the brain structure and function play a fundamental role in the development and neuropathophysiology of PLP[5,6]. However, the underlying neural correlates of how pain symptoms develop and how they can be alleviated in response to treatment remain unknown. This lack of information is mostly due to technical challenges and limitations associated with performing a given therapeutic approach within the constraints of a neuroimaging environment such as MRI[5,7,8].

Results from a number of studies attribute the development of PLP to maladaptive neuroplastic reorganization occurring within sensorimotor cortices, as well as in other areas of the brain. For example, it has been shown that following the amputation of a limb, there is a shift in the corresponding sensorimotor cortical representation of neighboring areas. As a result, neighboring areas apparently start invading the zones that used to correspond to the amputated limb[9,10]. In order to alleviate pain symptoms associated with PLP, treatments such as MT or motor imagery may be effective[9,11,12]. It is suggested that the alleviation of symptoms occurs putatively through the cross-modal re-establishment of afferent inputs, provided by the observation of mirror-reflected images from the nonaffected limb[12–17]. Through these images, participants are able to visualize the reflection of the opposite limb instead of the one that has been amputated, thus creating an illusion that both limbs remain. The illusion and immersive effects were previously studied by Diers et al. in healthy subjects in which a comparison of functional activation through functional MRI (fMRI) was evaluated after undergoing a task either with a common mirror box or virtual reality[18]. However, the neural correlates associated with the reversal of the maladaptive neuroplastic changes and the alleviation of symptoms remain poorly understood. Additionally, the underlying mechanism of PLP remains a topic of research as the clear underlying physiopathologic alteration behind the development of PLP is still incompletely elucidated while controversial findings have been revealed[5,19]. As stated above, multiple authors attribute the development of pain to deafferentation and cortical reorganization of the affected brain area (area of the amputated limb)[6–8]; however, opposite results were described by Makin and collaborators in which the presence of pain is associated with the preservation of brain structure and pain is attributed to a reduction interregional functional connectivity[19]. In view of these controversial and opposite findings, we believe that the novel approach presented here will bring additional relevant information to the study of PLP and will allow scientists to evaluate the effects of MT in a live environment with the degree of brain activation while comparing them with the levels of pain assessed in our full protocol[19].

Previous literature on this topic has shown that MT is one of the most appropriate behavioral therapies for the treatment of PLP due to its easy implementation and low costs[12]. In fact, previous studies of this technique have shown evidence of a reversal of maladaptive changes within the primary sensorimotor cortex in amputees with PLP[8,20,21]. Even though MT is perhaps one the most inexpensive and most effective approach to treat PLP[12,22–24], more studies are needed to confirm these effects since some patients do not respond to this type of treatment[8] and there is a lack of larger randomized clinical trials that provide high-evidence-based results[25].

One of the hypotheses by which MT can reduce PLP is related to the fact that the mirror image of the not-amputated body part helps to reorganize and integrate the mismatch between proprioception and visual feedback[26]. The underlying mechanisms of MT could be associated with the reversion of the maladaptive mapping of somatosensory[8,27,28].

For MT, subjects are required to perform several motor and sensory tasks using their intact limb (e.g., flexion and extension) while observing this effect in a mirror located in the midline of the participant's body, thereby creating a vivid and precise representation of movement within the area of the amputated limb[29].

To further develop the scientific understanding of the pathophysiology aspects involved in PLP, it is crucial to better characterize the underlying neuroplastic changes that result from limb amputations, as well as the improvement of pain symptoms provided by MT. In this regard, neuroimaging techniques, such as fMRI, have emerged as powerful tools to help elucidate the pathophysiologic mechanisms associated with cortical reorganization and provide clues toward optimizing the rehabilitation of individuals with PLP in the clinical context[30,31]. Furthermore, the high spatial resolution afforded by fMRI (as compared to electroencephalography, for example) allows for more accurate mapping of brain responses, such as finger and digit representations, in the sensorimotor cortex along with other regions of the brain[32].

To date, the neurophysiology associated with MT remains elusive due in large part to the challenges of carrying out the procedure within the scanner environment (i.e., it is difficult for an individual to perform the therapy while lying in the scanner). Here, we describe a method that allows for an individual to observe their own leg movement in real-time while lying supine within the narrow confines of the scanner bore. An accurate recreation of the vivid and immersive sensation elicited by the therapy can be recreated using a video camera that captures real-time images of the moving leg, and a system of mirrors and a monitor that can be viewed directly by the study participant.

Past studies have attempted to incorporate techniques such as video recording, virtual reality, and prerecorded animations as means to present the visual stimulus and circumvent these technical challenges[9,16,33,34]. Yet, these techniques have been limited in their effectiveness[35–39].

In the particular case of using a prerecorded video, there is an often poor synchronization between participants' movements and the ones provided by the video, as well as a lack of timing accuracy, which leads to a poor realistic impression that the individual's own leg is moving. In order to improve this sense of sensorimotor immersion, other techniques, such as virtual reality and digitized animations, have been attempted. Yet, they have failed to generate visually convincing sensations due to a low image resolution, a limited field of view, unrealistic or nonnatural human-like motions, and presence of motion lag (i.e., desynchronization of movement). Additionally, the lack of an accurate modeling combined with the poor control over other features, such as the effects of friction, momentum, and gravity, hinders the perception of a vivid and immersive feel[40]. Therefore, for amputees, it is worth to explore strategies to ensure that subjects are engaged in the cognitive task (observation) and immersive on the illusion of amputated limb movement. Finally, the required resources for developing and implementing these complex strategies may be time-consuming and/or cost prohibitive.

We describe a new approach that we believe creates a realistic and vivid sense of immersion whereby the participant can see a live and real-time video of a projected image of their own limb while they perform a session of the MT[31]. This approach is performed while the individual is lying in the scanner bore and is without substantial costs or extensive technical development.

This protocol is part of a National Institutes of Health (NIH) Research Project Grant (RO1)-sponsored clinical trial that evaluates the effects of the combination of a neuromodulatory technique, namely transcranial direct current stimulation (tDCS), with a behavioral therapy (mirror therapy) in order to relieve phantom limb pain[31]. We evaluate changes in the visual analog scale (VAS) for pain at baseline, prior, and after each intervention session. fMRI is used as a neurophysiologic tool in order to evaluate structural changes in brain function and its correlation with the relief of PLP. Therefore, an initial fMRI is obtained in order to have a baseline map of the structural organization of the participant's brain, which will either show that there is cortical maladaptive reorganization[5,6,8,11,13,14,18,28] or that there is not[19]; in the same way, the scientist can observe what areas are activated at baseline with the task of MT in order to understand the areas' activation response to the MT; lastly, it is possible to obtain a second fMRI postintervention to see if changes (modulation) have been generated in the cortical reorganization after the combined therapy with tDCS and MT and to analyze if those changes are correlated or associated with the degree of pain change. Therefore, this protocol allows scientists to evaluate structural reorganization changes in patients with PLPs during MT and also helps them to understand if these changes seen in fMRI are associated with changes in PLP, therefore providing additional details on how MT affects brain structural and functional activity to modify phantom pain.

PROTOCOL:

1. Preparation of the subject

1.1. Prior to participation, have the participant complete a consent form and an MRI safety screening evaluation, the latter carried out by the neuroimaging technician at the scanning facility, to ensure that the participant does not have any known contraindications to being scanned (e.g., metal in their body, a history of claustrophobia, or pregnancy).

1.2. Provide the participant with detailed instructions with regard to the experimental procedure.

1.3. Have the subject listen to an instructional recorded audio to ensure that they are able to understand and follow the instructions provided during the scanning procedure.

1.4. Carry out a practice run in a mock scanner to facilitate the familiarization of the task instructions within the scanner environment.

NOTE: The mock scanner is similar in every way to the real data-acquiring MRI scanner, but without the active magnet.

1.5. Give clear instructions to the participant to avoid any movements of the residual and phantom limb to avoid any contractions of the stump muscles that can interfere with the brain signal.

2. Preparation of the experiment

NOTE: The experimental protocol is similar to what has been previously described for the purposes of investigating the neural correlates associated with the mental imagery of moving the upper limbs. Here, we have adapted the approach to the movement of lower limbs. Specifically, the behavioral tasks consist of the following.

2.1. Before entering the scanner room, ask the participant to remove their prosthesis and any metal objects.

2.2. Have the MRI technician make sure that the participant **has no metal on their body that might put them at risk.**

2.3. Transport the participant to the MRI room in an MRI-compatible wheelchair; after that, ask the participant to transfer themselves to the MRI scanner bed.

2.4. For the MT, comfortably place a single-piece, MRI-compatible, horizontal mirror (10,000 mm x 255 mm x 3 mm) supported by a triangular stand between the legs of the participant while they are lying supine on the scanner bed. Use sandbags in order to allow stability and a better positioning of the mirror. Attach the mirror stand to an adjustable arm so that it can be positioned in accordance with the subject’s height and positioning without contacting any part of the body (**Figure 1**).

[Place **Figure 1** here]

2.5. For the visual feedback, mount an MRI-compatible digital camera on an adjustable tripod stand near the intact leg of the participant (**Figure 1**).

NOTE: The camera used is listed in the **Table of Materials** and costs approximately 217 USD. The camera acquires images in 1,080 pixels image resolution. Since the camera itself was not placed inside of the MRI bore, there is not a need for costlier MRI-compatible systems. The camera is attached to an MRI-safe IV pole via a gooseneck modular hose to enable positioning changes.

2.6. Attach the camera to a tripod, allowing the appropriate adjustment of the viewing angle and field of view.

2.7. Place a second mirror on the MRI head coil, allowing the participant to view the image presented on the monitor directly while lying completely inside the scanner bore (**Figure 2**).

[Place **Figure 2** here]

2.8. Set up the real-time video image transmission to be sent through a computer-controlled system and project it onto a monitor placed at the back of the scanner bore (near the head of the participant).

NOTE: There is no perceivable time delay between the projection and the captured actual movement. The actual movement and the visual feedback are separated by less than a second which does not interfere in the real-time feeling, as stated by participants.

3. Scanning and data collection

3.1. Acquire fMRI data with a 3 T scanner using an 8-channel phased-array head coil.

3.2. Obtain imaging sequences that include a high-resolution T1-weighted structural image (TE: 3.1 ms, TR: 6.8 ms, flip angle: 9°, isotropic 1 mm voxel size) (anatomical scan), and blood-oxygen-level-dependent (BOLD) fMRI signal measurements using a protocol based on multislice gradient (fast-field) echo-planar imaging (EPI) and standard parameters (TE: 28 ms, TR: 2 s, flip angle: 90°, isotropic 3 mm voxel size, oriented axially and covering the whole brain).

NOTE: The entire scanning procedure lasts approximately 30 min. This includes an initial 4 min structural (anatomical) scan and four task (functional) acquisitions lasting 6 min each. For each task (functional acquisition), the patient is expected to tap their foot at a speed of 1 tap every second.

3.3. During the scans, have the participant wear sound-isolating MRI compliant headphones (e.g., Westone) throughout the scanning session to hear the investigator's auditory commands.

3.4. While the patient is lying in the scanner, play the auditory track so that the participant hears a series of auditory cues for performing the given behavioral task.

3.5. Use the following commands: 1) “leg” for movement of the amputated leg (see the note after step 3.11); 2) “mirror” for movement of the intact leg while viewing a real-time video recording (thus observing the movement of a leg in the position of the amputated leg using the mirror); 3) “rest” in which the participant stops any leg movement and lies motionless with their eyes closed. In addition, have the investigator says “start” and “end” to signify the beginning and the end of the experimental run, respectively (**Figure 3**).

[Place **Figure 3** here]

3.6. Have the participant perform a movement with the nonamputated lower leg with the eyes closed (i.e., repeated plantar flexion and dorsal flexion of the foot at a pace of approximately one tap per 2–3 s).

3.7. Have the participant perform the same leg movement, but now the participant observes a mirror image of his/her leg moving in the place of the amputated leg using real-time video capture of the movement of the intact leg.

3.8. Have the participant perform a rest condition, in which he/she lays still with no movement of the legs.

NOTE: Each condition lasts for 20 s (i.e., one experimental block = 60 s) for a run length time of 6 min (six repetitions of the experimental run per block).

3.9. Collect data in a single session for each participant.

3.10. Instruct the investigator to take note of any unwanted movements, and, in between the runs, to instruct the participant to keep the correct pace and movements.

3.11. Make sure that, after the procedures are performed, the investigator transfers the data to an encrypted flash drive and stores it in a secure location in the facility.

NOTE: In this protocol, the word “leg” is used in place of the word “foot”. Even though the participants are only making foot movements (due to restraints from the MRI machine), most of them have a bigger part of the lower limb amputated and are referred as leg amputees, not foot.

4. Analysis

4.1. Analyze the functional neuroimaging data using standard techniques[30,41], using the longitudinal analysis design (baseline and posttreatment) and processing stream in the FMRIB Software Library (FSL) software package[42,43].

4.1.1. For each functional scan, perform 3D motion correction using the first volume alignment, high-pass filtering to remove temporal linear trends, and perform a correction for slice time acquisition and spatial smoothing (Gaussian kernel, 5.0 mm full width at half maximum [FWHM]).

4.1.1.1. Mark volumes with a motion above 0.9 mm in any direction with FSL's motion outlier detection processing stream and mathematically "scrub" them from the final analysis[44].

NOTE: If more than 25% of the volumes are designated for removal, the whole acquisition should be excluded from the total dataset.

4.1.2. Coregister each of the preprocessed functional images to the high-resolution anatomical and, then, bring them into standard Talairach space.

4.1.3. Fit a general linear model (GLM) to a voxel time course where each experimental condition is modeled by a boxcar regressor that should be smoothed with double-gamma hemodynamic response function.

4.1.4. Use the high-resolution anatomical T_1-weighted anatomical volume to construct an inflated cortical surface mesh to see the sulcal activation, and then, project individual subject maps for each contrast of interest onto the subject's reconstructed mesh.

NOTE: The projections should show the significant values from the GLM. Set the statistical significance value threshold at the standard criterion of $p < 0.001$ corrected for multiple comparisons, using a cluster-size threshold adjustment.

4.2. Conduct a region of interest (ROI) analysis.

4.2.1. Define the primary ROI broadly with FreeSurfer's Desikan atlas[45] of the primary sensorimotor cortex and, then, refine it for each subject by using the subject-specific functional activation during the leg vs. rest condition at the baseline scan.

4.2.2. Reflect the refined primary ROI onto the homologous area of the opposite hemisphere (i.e., ipsilateral primary sensorimotor representation of the intact lower limb).

4.2.3. Use the standard FreeSurfer anatomical Desikan atlas[45] to define the entire (bilateral) occipital visual cortex for the secondary ROI.

REPRESENTATIVE RESULTS:

Generating the sensation associated with MT using real-time video projection is feasible. Participants have subjectively reported that the video image perceived is life-like and the sensation is immersive.

Furthermore, the patterns of cortical activation associated with MT (i.e., movement of the leg and viewing the projected mirror image) in the scanner environment are robust. In a pilot study, the cortical responses to MT were recorded using fMRI in a participant with lower-limb amputation of the left leg (male, 56 years old, traumatic amputation of lower leg below the knee) following the task protocol described above. Comparing leg movement versus the rest condition resulted in a robust activation within the sensorimotor representation of the leg of the contralateral (i.e., left) hemisphere. Ipsilateral cortical activation was observed within the sensorimotor leg area (**Figure 4A**). The mirror condition versus rest condition also confirmed robust contralateral as well as ipsilateral activation of the cortical leg sensorimotor representation. Additionally, robust cortical activation was seen with posterior occipital (i.e., visual) cortical areas associated with viewing the projected image of the moving leg.

The patterns of activation described represent activations at the baseline condition, that is, at the initiation of the therapy period. The initial responses serve to define the baseline activation for the purposes of defining regions of interest (ROIs), and a subsequent comparison after the MT protocol is completed in each individual.

[Place **Figure 4** here]

FIGURE LEGENDS:

Figure 1: Video camera and mirror set up. The mirror is positioned between the legs at an angle of about 45°, depending on the participant's height and amputation level. The goal is to cover the residual limb and make it invisible to the video systems. Sandbags are used to keep the mirror in the correct position. The camera positioning is also adaptable and can be easily changed using the tripod or the adaptable hock (changes the angle of the camera).

Figure 2: Schematic of video camera and image projection in the scanner environment. The real-time video projection of the mirror therapy system consists of three subsystems. 1) Camera and monitor subsystem. The video is transmitted to the monitor, so the subject can watch the leg and mirror leg movements in real-time. 2) The head coil with the mirror attached. The mirror in the head coil allows the participant to watch the monitor without moving their head. The mirror is at a 45° angle at eye level. 3) The mirror and sandbags. The MRI-compatible mirror is carefully placed between the legs and the residual limb in a way that it covers the residual limb and allows for the best image to be shown.

Figure 3: Task design. The task design consists of three steps. During the first "leg" step, the subject is instructed to move the leg (flexing the foot) at a pace of about one movement every 2 s (10 movements in 20 s), with their eyes closed. For the second "mirror" step, the participant has to keep moving the leg (10 movements in 20 s) while looking at the video monitor displaying the online real-time mirror image of the legs. The last step instructs the subject to rest.

Figure 4: Representative example of the cortical activations in response to mirror therapy in the MRI scanner. (**A**) Comparing leg movement versus the rest condition resulted in a robust activation within the sensorimotor representation of the leg of the contralateral (i.e., left) and ipsilateral cortex. (**B**) The mirror condition versus rest condition also confirmed a robust

contralateral and ipsilateral activation of the cortical leg sensorimotor representation, as well as occipital (i.e., visual) cortical activation associated with viewing the projected image of the moving leg.

DISCUSSION:

This protocol describes a novel, feasible procedure that allows investigators to accurately characterize the neural correlates associated with MT in individuals with PLP.

As previously mentioned, past studies have attempted to investigate the neural correlates associated with MT treatment by incorporating various techniques such as video recording, virtual reality, and prerecorded animations[9,33,34]. However, these approaches have been limited in terms of effectiveness[37–39]. In the protocol outlined here, we incorporate simple, commercially available, and low-cost elements to create a life-like and immersive sensation associated with MT within the MRI environment. All the equipment used is MRI compatible (i.e., nonferromagnetic materials) and can be easily adjusted and modified for each individual. The key elements consist of three main subparts: (1) video camera and monitor; (2) the reflective mirror attached to the head coil; (3) the large reflective mirror and supports. The video is transmitted to the monitor so the subject can watch the leg and mirror leg movements in real-time. The orientation of the mirror in the head coil allows the participant to view the monitor while lying supine and without excessive movement of the head. The mirror is adjusted to the subject's amputated leg length using an adjustable stand in order to avoid any contact with the participant's leg. From a data acquisition and analysis point of view, functional neuroimaging data is analyzed using standard techniques (i.e., region of interest analysis) with special emphasis on a pre-post longitudinal design[30,41].

Besides the real-life immersive sensation provided to the participant, another advantage of this protocol is that the system can be adjusted for the purposes of viewing different limbs (upper and lower) and can be used to test any combination of limb movement.

The immersive sensation provided by the video transmission is an important factor when it comes to generating the potential therapeutic effect of MT. The use of the real-time video captured from the video camera as presented here can be superior to past approaches such as computerized images, virtual reality, or prerecorded images. However, we did not compare this technique with visual illusion ones. Moreover, a previous study in healthy participants evaluated functional brain activation after performing a task with a conventional mirror box and a virtual-reality-projected image of the upper limb. In the results of this study, Diers and collaborators found no differences between vividness or perceived authenticity of the illusion between the visual reality illusion and the mirror box therapy[18].

On the other hand, this protocol also has its limitations and challenges associated with it: due to the nature of the leg movement, motion artifacts (i.e., associated with excessive head movement) may compromise data quality. Although the patient is allowed to see a projected live image of their own limb, the protocol lacks a questionnaire to properly assess the vividness and immersion that the participant feels while undergoing the tasks. In addition, we did not compare the task performed in this technique with other strategies, such as visual stimuli only of a recording of the leg movement without the patient actually performing the movement or a virtual reality imaging projection of a lower limb moving. This was done in particular because it was not the goal of this protocol and because there are previous studies that have already studied and compared these interventions and revealed no difference in the pattern of activation, as well as no difference in vividness of the task between interventions, as mentioned above[18]. In addition, to overcome challenges related to the motion, we employed current state-of-the-art motion detection and correction strategies[26]. To further improve data quality, new strategies (e.g., physical restraints placed around the subject's hips to help isolate leg motion) are being pursued. Lastly, with regard to modification and troubleshooting, we initially had a fixed camera stand that did not allow us to obtain and adequately capture the patient's lower limb reflection in the mirror; however, utilizing an adjustable stand, we were able to obtain the most precise and accurate image transmission. Additionally, during the first steps of the development of the protocol, the mirror stand was fragile and fell easily with any mild movement. This was overcome when sandbags were added to give stability to the mirror montage.

Finally, given the ease of implementing the experimental setup, this approach may allow the evaluation of the effects of MT not only in limb amputees but also in other conditions which use this treatment approach, such as stroke and spinal cord injury.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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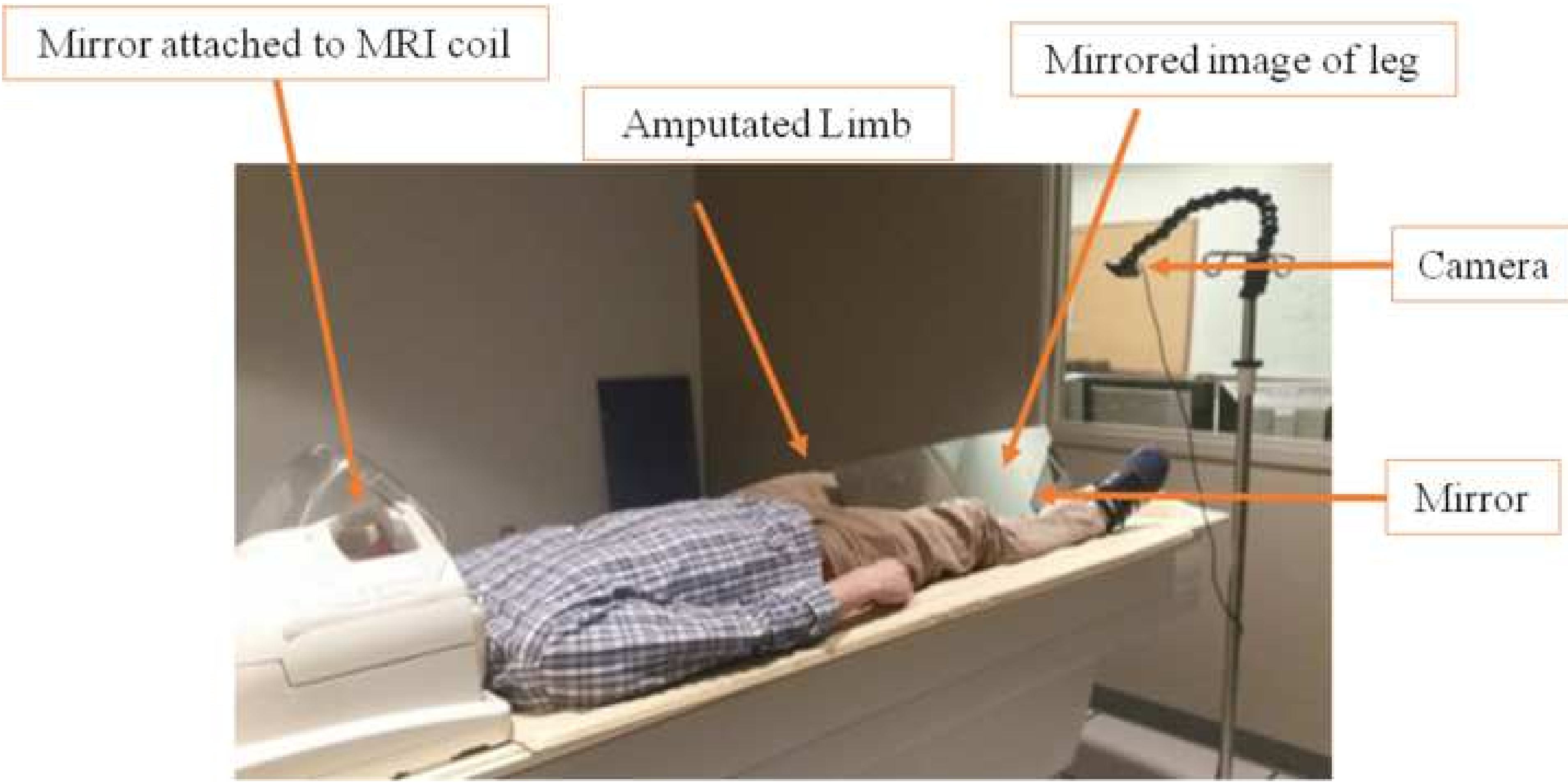


Figure 2

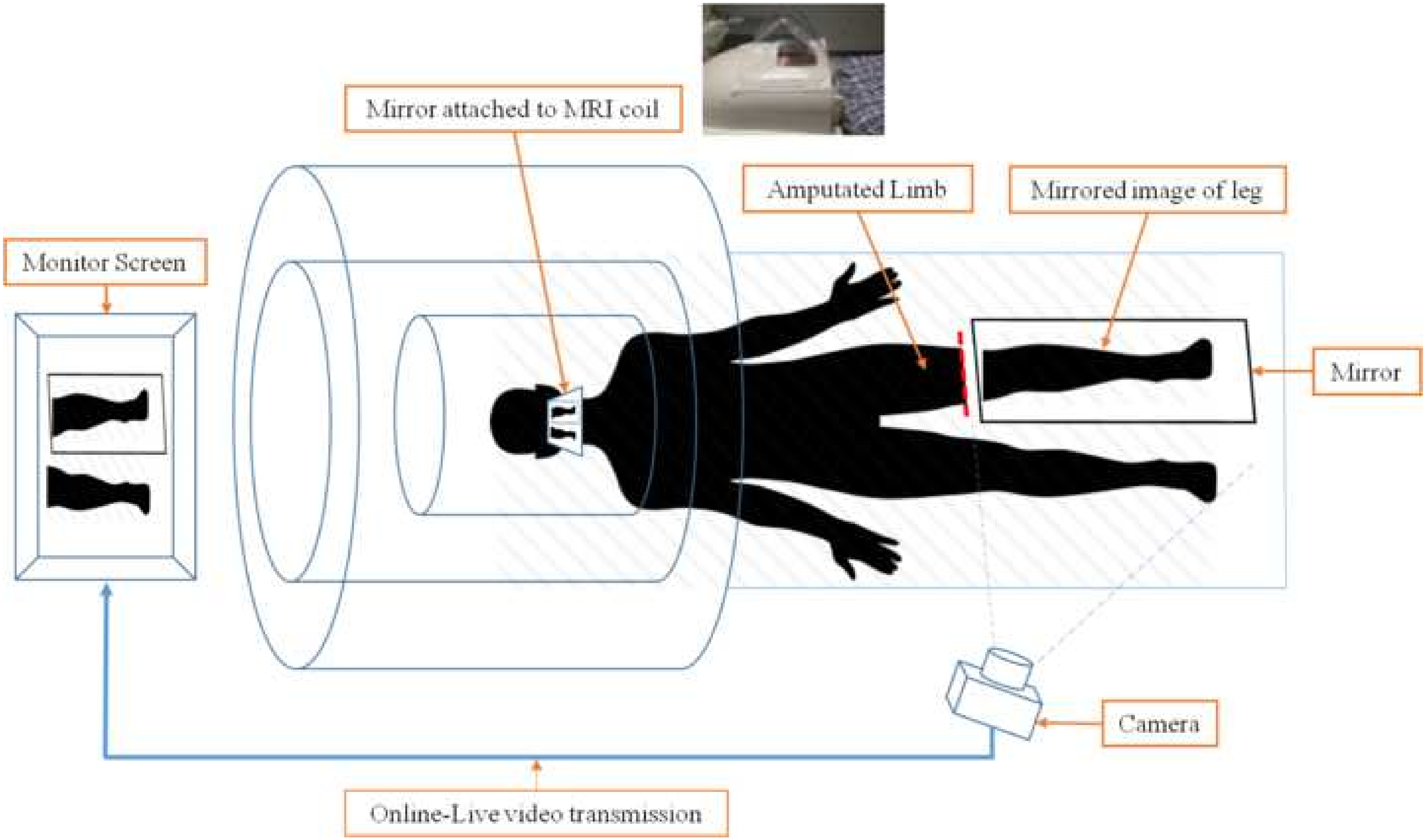

Mirror attached to MRI coil
Amputated Limb
Mirrored image of leg
Monitor Screen
Mirror
Camera
Online-Live video transmission

Figure 3

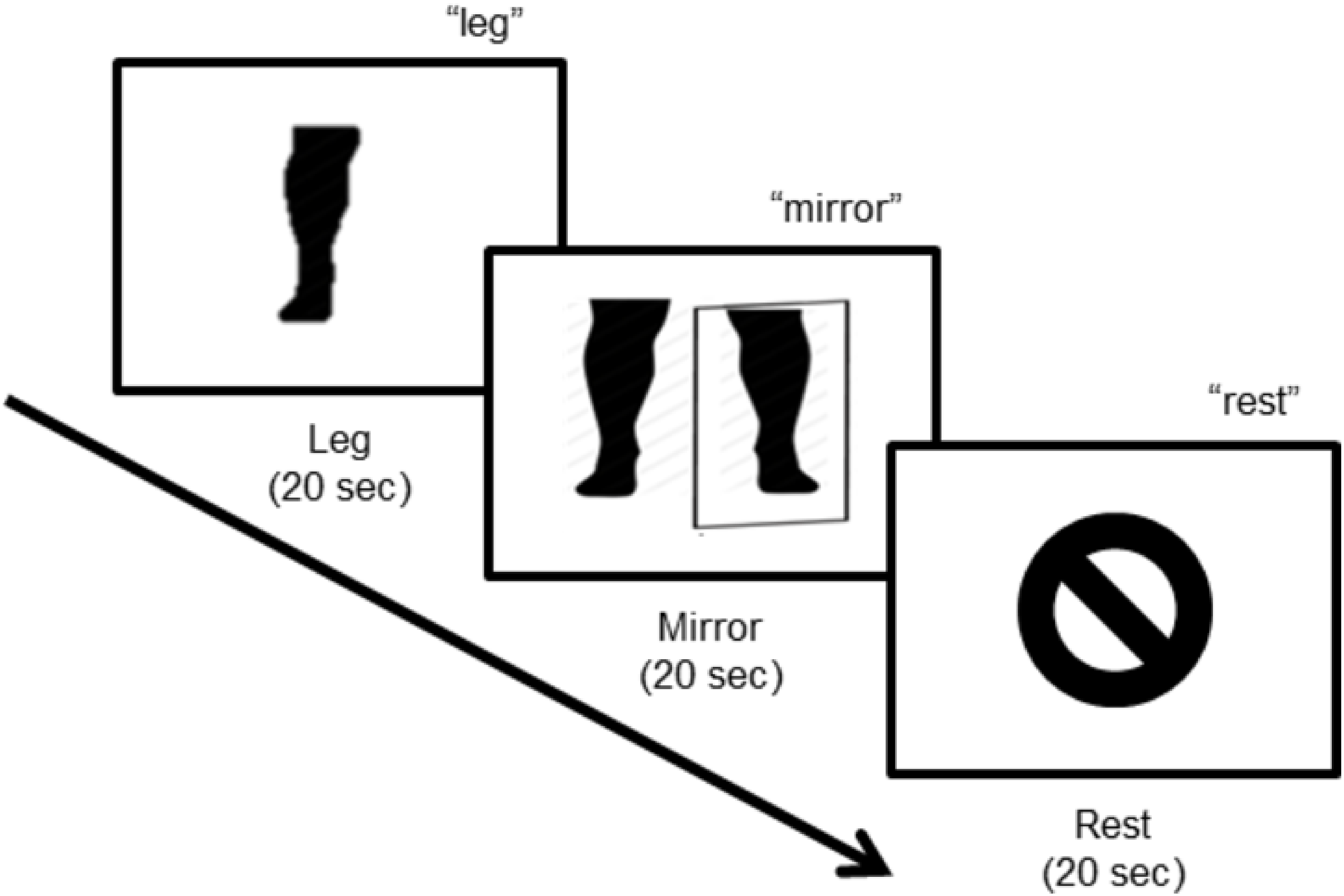
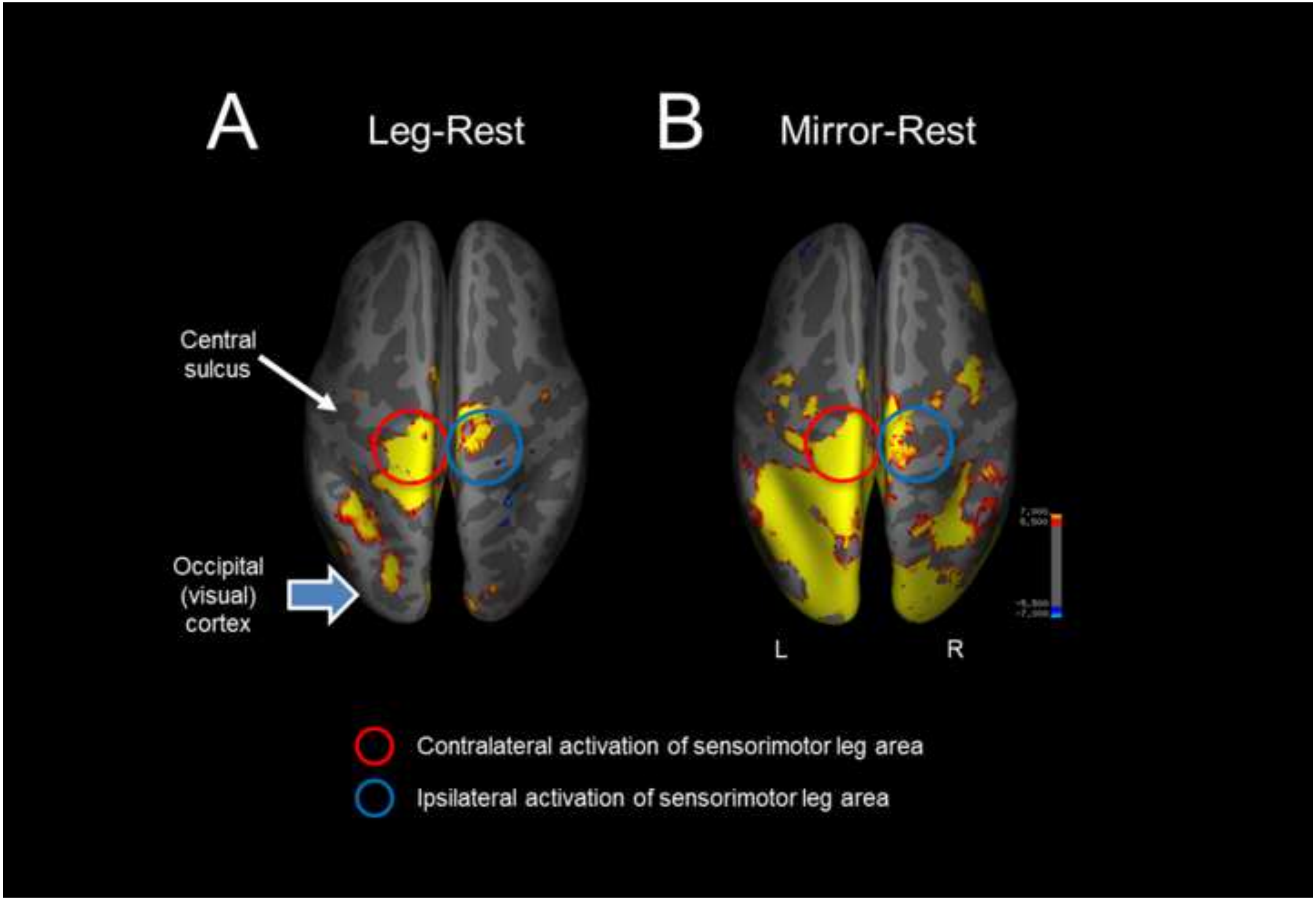


Figure 4


A
Leg-Rest
B
Mirror-Rest
Central sulcus
Occipital (visual) cortex
L
R
Contralateral activation of sensorimotor leg area
Ipsilateral activation of sensorimotor leg area

Name of Material/ Equipment	Company	Catalog Number
Scanner	Phillips	NA
Camera	Logitech	NA
Monitor	Cambridge Research Systems	NA
Mirror	TAP Plastics	99999
Mirror stand		NA
Headphones	Westone Sensimetrics	PN 79245
MRI Scanner	Phillips	3.0 T Philips Achiev

Comments/Description
3 Tesla Philips Acheiva MRI scanner
HD Pro Webcam C910
3D BOLD screen for MRI
Mirrored Acrylic Sheets (Cut-to-Size) - Clear 1/8 (.118)" Thick, 10" Wide, 40" Long
Mirror stand was built by the co-investigators from a rectangular piece of wood
Replacement comply foam tips for universal-fit earphones. Canal size: Standard 6 pieces/ 3 pair MR compatible in ear headphones
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Answer: This is template language for fMRI processing. It is the established FSL pipeline used in the analysis of this protocol, therefore this is what was done using the proper terminology and sequence. We adjusted the text as best as we could to also keep the enough details for the reproduction of the procedure.

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1) modifications and troubleshooting → a modification and troubleshooting were addressed in the manuscript
2) limitations of the technique, → A limitation paragraph was added
3) significance with respect to existing methods, → We have stated the significance with respect to other methods.
4) future applications → We discuss further application of this protocol as a tool for understanding the underlying pathophysiologic mechanism of other neuropsychiatric disorders such as spinal cord injury, stroke among others.
5) critical steps within the protocol. → The critical steps are detailed in the protocol section.

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Answer: We have reviewed all figures and they all have the corresponding legend.

• **References:**
1) Please use superscripted numbers (e.g. edit line 132).

Answer: We have fixed this reference.

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-

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Comments from Peer-Reviewers:

Reviewer #1:

This experiment setup is expected to contribute to research for brain activity in mirror therapy. Also, it has potential to be applied to case studies. There are some comments, please consider.

Major

Introduction
1. L55-57: You should mention the point of similarity and difference with previous study which was conducted by Diers (Brain Res. 2015) to suggest novelty of your system.

Diers M, Kamping S, Kirsch P, Rance M, Bekrater-Bodmann R, Foell J, Trojan J, Fuchs X, Bach F, Maaß H, Cakmak H, Flor H. Illusion-related brain activations: a new virtual reality mirror box system for use during functional magnetic resonance imaging. Brain Res. 2015 Jan 12;1594:173-82.

Answer: Thank you for pointing this out. In fact, we consider the work of Diers et al. as an important influence in our work, and we now have added details outlining similarities and differences of this publication compared to our study. One important issue is that Diers et al. study evaluated brain responses in healthy subjects, while we are evaluating patients with phantom limb pain with associated evidence of impaired neuroplastic reorganization prior to treatment. In addition, Diers et al. evaluated the upper limb while we are exploring neuroplastic reorganization associated with lower limb training. In order to evaluate therapy implicating the lower extremity, we had to develop a novel protocol that included the use of a camera transmitting real time images of the subject's moving limb. As discussed by Diers et al., there are inherent limitations and challenges in using a mirror-based system alone to make the arm movements seem more natural. This required the use of a complex system of mirrors. However, as stated by our participants, viewing the video transmission made the feeling of movement very real.

Protocol

2. L132-136: Regarding the condition 2), did the experimenter ask the patient to move his phantom leg simultaneously with his intact leg?? Did his muscle around stump contract during seeing mirror visual feedback?? I think that these factors could influence the brain activity.

> **Answer**: We did not ask the patient to move their phantom leg simultaneously. The whole point was that the patient was not moving the affected limb in order to avoid signaling from the muscles from the residual limb. In fact, we were clear to specify to the participant to only move the unaffected leg in both conditions (eyes open and eyes closed). Thus, we do not expect any influences on brain activity. A phrase clarifying this instruction has now been included in the protocol.

3. L154-172: I do not think that the actual position of phantom limb spatially matched with mirrored phantom limb. You should describe the detail of method to minimize the discrepancy between actual position of phantom limb and mirrored phantom limb.

> **Answer**: There were no methods to assess or match the actual position of the mirror leg to the phantom limb since this was not the goal of this protocol. The goal was to use mirror therapy to give the illusion of having an intact full moving limb. Besides that, most of the amputees do not have phantom limb sensation all the time. The majority of amputees have these sensations a couple of times a day, and sometimes the phantom sensation is in a non-physiological position. In order to match the position, we would have to guarantee that the subject had an ongoing phantom sensation and that the phantom was in a physiological position that could be reproduced. Therefore, we do not recommend adding this step in this protocol.

4. L154-172: Was there time-delay between actual movement and visual feedback?? You should about time-delay because the video transmission could make time-delay among them.

Answer: The image was transmitted in real time from the camera directly to a monitor. There is no perceivable time delay between the projection and captured actual movement. The actual movement and the visual feedback are separated by less than a second and did not interfere in the real time feeling as stated by our participants.

Result

5. L238-239: The result of cortical activity in posterior occipital with viewing the projected image of the moving leg was very interesting. You should discussion the brain activity referencing the previous report (Preißler et al. 2013) in a concise description.

Preißler S, Dietrich C, Blume KR, Hofmann GO, Miltner WH, Weiss T. Plasticity in the Visual System is Associated with Prosthesis Use in Phantom Limb Pain. Front Hum Neurosci. 2013 Jun 24;7:311

Answer: Thank you for pointing this out. Indeed, the results of Preißler et al. are very interesting and relevant. In our protocol, visual cortex activation is expected and related to the task the participants are performing. The figure presented in the manuscript is comparing the Movement condition with the Mirror condition. During the movement condition (MOV), the participants move their leg keeping their eyes closed. In the mirror condition (MIR), they continue to move the leg but with the eyes open. Therefore, activation of the visual cortex is expected due the differences in the task being carried out (eyes open vs. eye closed).

Discussion

6. You mentioned the technical problems in previous studies which using only video feedback of limb movement. You should have compared the brain activity between condition of mirrored visual feedback and the condition of simple video feedback. You should describe the lack of experimental procedure as limitation and future study.

Answer: The limitation of previous studies using video feedback is now discussed. The greatest limitation is that this approach does not provide the feeling of immersion. However, as mentioned, there is a lack of studies comparing the methods of visual illusion with Mirror therapy or mental imagery. Diers et al. showed no differences between visual illusion and mirror box therapy, showing that both techniques may be comparable in providing the illusion we are looking for in this protocol. As the primary goal of this experiment is to better understand the neuroplastic reorganization of participants undergoing mirror therapy, we do not think that a simple video feedback condition is necessary. We have added a comment in the limitations section regarding the lack of experimental procedures as we did not compare brain activity between the condition of mirrored visual feedback and the condition of simple video feedback.

Minor

7. L131: The is typo in reference [56]

Answer: We have fixed this typo.

Reviewer #2: --
Manuscript Summary:
This article present a protocol to study limited aspects of mirror therapy using fMRI.

Major Concerns:
The authors are not specific enough with what they want to investigate with this set up. I understand their intention is to merely present a protocol and the way they overcame the challenge of having a patient performing mirror therapy in a MRI scan (more on that below). However, every protocol is devised to answer a specific research question which is not clearly stated, nor the protocol is wide enough to answer a range of different questions.

It is unclear to me if the authors want to simply see the activation arising from observing a limb movement from a first-person perspective through a mirror or if they want to answer a question about pain. In the first case they should state how this would advance our understanding and give a better overview of similar research of neuroimaging. In other words, there is not much novelty in showing that with mirror feedback, ipsilateral activation of the sensorimotor cortex is elicited, and there is plenty of evidence for that. If the aim is instead investigating pain, then the authors should give better account of the literature, at least the one on fMRI and PLP (i.e. Jutzeler 2015) and express clearly how this protocol would contribute to improve our understanding. Furthermore, the protocol itself is not sufficient to answer any question about pain as they do not include in their methods any plat to assess that. A fMRI paradigm can be hardly separated from this kind of evaluation, which is also integral part of the experimental protocol.

> **Answer**: The goal of this protocol is to describe an approach to perform mirror therapy inside an fMRI scanner. This is entirely in-line and of the scope of this journal by outlining experimental details and methods designed to investigate the structure, function, physiology, and pathophysiology of the nervous system. Here, the technique is described using amputees that have PLP. Mirror therapy is remains commonly used in these patients, even though its underlying neurophysiological mechanisms are not yet completely elucidated.
>
> Furthermore, this experimental procedure can be applied for other conditions that also rely on the use of mirror therapy. This includes conditions such as pain related to stroke and patients with de-afferentation pain.
>
> The novelty of the approach is as follows: previous studies have applied mirror therapy or mental imagery to revel associated brain activation patterns only. Here, we have the opportunity to observe changes in neuroplasticity and how there is functional

reorganization after an amputation. Using this experimental protocol, several lines of investigation can be pursued such as comparing cortical reorganization before and after a given treatment, or to correlate changes in brain activation with the degree of phantom pain and other baseline characteristics of the participants.

In our case, this protocol is part of a much larger clinical trial comparing multiple treatment modalities in amputees with PLP (NCT02487966). The goal of this trial is to compare changes in brain activation before and after treatment and correlate findings with pain levels.

In addition, most of the available literature has evaluated upper limb function, which is much simpler to perform technically and the homunculus representation of the hand area is much bigger. This is contrary to that we are investigating in our population (i.e. lower limb amputees) which has been less studied despite the fact that the number of individuals with PLP associated with lower extremity amputation is extremely large. This makes our protocol helpful for general applicability as well as for other disorders such as spinal cord injury and stroke.

R87-94 Authors talked about challenges of mirror therapy in MRI scan but they do not clearly state what they mean with that. It seems that the challenge is to perform the movement inside the limited space of the scan. However this is not much of a challenge for the lower limb (the challenge for the lower limb is to perform movements without moving excessively the head and they do not tackle this problem in any way). Afterwards they vaguely imply that the challenge is the accurate recreation of the visual feedback, even though I do not recall studies of mirror therapy in fMRI scan highlighting such difficulties (i.e. Foel). To show limitation of previous studies they use unacceptable references:

Answer: We thank the reviewer for pointing this out. We have now clarified the text with regard to the challenges of performing the mirror therapy within the constrained space inside the scanner. That is, the challenges associated with moving the leg, viewing this movement, and not moving the head. Previous studies, like the one mentioned (Foel), were designed to evaluate changes in cortical mapping using fMRI with upper limb movement and using a mirror placed while subjects performed hand movements observed in real time using a head coil mirror. This was more difficult as they needed extra devices to make the image of the hand appear in the appropriate place on the body. In our protocol, there is no need for additional mirrors or camera systems because for the lower limb, the movements are observed in real time by direct video transmission to a monitor placed at the back of the scanner. However, due to the positioning and space constraints of the MRI machine, a single mirror was used to allow subjects to watch their leg movements. Therefore, the need of this protocol, in which a camera and video system are used to give real time visual feedback is highly relevant and appropriate. This issue is further supported by adding further citations revealing the limitations of previous study protocols.

*R97 reference 23 is not a proper reference for a peer review article and does not proof how mirror therapy is limited in effectiveness

> **Answer**: This reference was deleted.

*R97 I haven't read reference 24 but from a quick glance at the paper is seems completely out of place. Doesn't seem to provide any evidence that previous attempt at mirror therapy in a MRI scan were limited in effectiveness. What do you mean with effectiveness anyhow?

> **Answer**: In this protocol, we discuss why using a real time camera video image is superior to using virtual reality. Our approach provides an accurate and real visual feedback and immersive sensation of the mirror therapy. The reference 24 explains why egocentric distances (i.e. the apparent distance the subject views the object seen in a virtual reality environment is not well perceived) thus further supporting our statement that a real live online live video projection of the subjects' own leg is better than using a virtual reality imaging of a non-real leg. We have modified the text to make this point clearer.

R102-104 The authors overstated the importance of realistic visual feedback for PLP relief (especially in terms of image resolution), without proper support for this idea

> **Answer**: The goal of mirror therapy is to give accurate visual feedback of the missing part of the limb. Only a few studies have compared this technique with other approaches such as virtual reality. Even though, so far, there is no data comparing the efficacy versus image resolution and/or accuracy. The proposed mechanism underlying mirror therapy effects take into consideration the immersive feel provided by real time visual feedback. Therefore, we consider that a realistic visual feedback has a direct effect on the experimental results. We have added more references with regards to the importance of realistic visual feedback and PLP relief.

The authors present ideas on PLP in the introductions as facts, which is not correct, such as "The alleviation of symptoms occurs putatively through the crossmodal re-establishment of afferent inputs, provided by the observation of mirror-reflected images from the non-affected limb". It is not certain that is the reason why alleviation of PLP happens. The whole introduction should be revise to be more clear and what we know is more certain than hypotheses.

> **Answer** :This is one of the possible mechanisms and the hypothesis in which we have based our on-going clinical study. We have modified the introduction accordingly to clarify that there are other hypotheses given that the neurophysiological mechanisms are still not well elucidated.

Minor Concerns:

A figure showing the view of the set up from a patient point of view would be helpful.

Answer: Unfortunately, taking areal picture or filming of the mirror/monitor inside the scanner is not be feasible due to space constraints and safety issues (i.e. it is not possible to enter with a camera inside the head coil space).

R68 It is unclear what "this reversal" is referring too.

Answer: The term “this reversal” refers to the reversal of the maladaptive plasticity theory explained in the same paragraph. We modified the phrase to make this point clearer.

R70-77 Could be a good place to mention limitations of mirror therapy in the treatment of PLP.

Answer: We modified the introduction to include more support for the use of mirror therapy and its limitations.

R84-86 Indicate the authors aim to assess correlates of pain relief, and they use the spatial resolution superiority of fMRI as reason to choose that instead of EEG for example. This means however that they are already assuming that the pathophysiology of PLP is completely expressed in the somatosensory and motor cortices, which is reductive.

Answer: One of the most accepted models to explain PLP is related to the reorganizational changes and maladaptive plasticity seen in amputees with pain. In addition, there are several studies that show that altering this disorganization is associated with improvement in pain symptoms. Therefore, we are basing our study on available literature. However, we recognize the existence of other theories. In order to address the concern of the reviewer, we have added a statement in which we acknowledge that there are other mechanisms that can be associated with PLP such as Makin et al. Additionally, as stated in the introduction, we state that the exact underlying mechanism of PLP is not well elucidated. Therefore, that is why we believe that this protocol will help to clarify this issue. However, our line of research focus is in investigating these changes in an patient population that has been less studied (i.e. lower limb amputees). We acknowledge that pain causes other neurophysiological alterations that can be better addressed such as EEG or TMS. However, in this case, we are focused on characterizing changes seen in cortical and somatosensory areas.

R68 The authors talk about the reversal of maladaptive plasticity as if it was already mentioned.

Answer: The sentence was modified accordingly to clarify what the term reversal is referring to.

R87 The opening sentence is odd

Answer*:* The sentence was modified accordingly: New sentence now reads as follows: “Phantom Limb Pain (PLP) refers to the sensation of pain perceived within the area corresponding to the missing limb post amputation”.

R131 reference [56] does not exist in the bibliography

Answer: This reference was not linked in the reference manager. This is now corrected.

R133 There a more unambiguous way of calling the movement is plantarflexion/dorsalflexion.

Answer: This is the most accurate terminology used in the field. However, to avoid any possible misunderstanding, we have added explanations of the movements between parenthesis.

R148-150 are there any instruction given to the patients to rule out the possibility that they are not actually moving the phantom limb during mirror feedback?

Answer: There are clear instructions given to the participants, so they do not move the phantom and residual limb in order to avoid any movement of the stump that can alter the signal on fMRI. To clarify, a sentence was included in the instructions section:

“Give clear instruction to the patient to avoid any movements of the amputated limb as to avoid contraction of stump muscles that can interfere with the brain signal”.

R226-227 do the subject spontaneously report about the VR immersions or is there an interview? How many patients stated this? How many people out of the total number that underwent the paradigm?

Answer :There is no interview or questionnaire carried out. However, in our protocol we do not have any VR, therefore there is no VR immersion. However, if the question is in regards of immersion after our intervention, most of the patients reported immersion, including during protocol setup in which we used healthy controls to test the experiment set up. We have added a sentence in the limitations section that states that we did not have a questionnaire to assess either VR immersion or immersion after our intervention. However, is recommended to use one in further studies.

Reviewer #3:

Manuscript Summary:
This manuscript introduces a protocol to study the neuronal correlates of mirror therapy in the MR scanner environment. For this purpose, a combined mirror-camera-setup is introduced that allows amputees while lying in the scanner to see their moving (intact) limb. In the here

introduced protocol, the authors compare a situation where the patient is asked to move the intact limb and to close the eyes, to move the intact limb and to look at the mirror image of the intact limb, or to rest. These three conditions were compared with respect to the BOLD signal change they trigger in sensory cortex. The protocol is of potential interest to researchers studying the neuronal basis of phantom limb pain. However, there are a few serious flaws of the protocol the authors should acknowledge.

Major Concerns:

-The authors write repeatedly in the introduction and discussion that the used set-up is cheap, and easy to construct. This is in fact not true. I have myself worked with a similar online-camera system in the MR-scanner. MR-compatible cameras are very expensive. The model I bought was around 7.000 Euros. I would therefore not consider a setup requiring an MR-compatible camera as cheap or easy at all, because a specific camera has to be bought, or has to be self-constructed, which can be challenging. I would therefore ask the authors to please 1) remove the statements from the manuscript that their setup is cheap, and 2) add details to the exact camera they used, including a link to the website.

> **Answer** :We modified the text accordingly, explaining that the camera is not a relatively expensive piece of equipment. The camera used is a Logitech HD Pro C910 with a current cost of approximately $217.00 USD (please see the following link https://www.amazon.com/Logitech-Webcam-C910-Cameras_Frames/dp/B003M2YT96). For safety concerns, we removed all ferrous metal components. This was mostly just the small stand that was attached. Since the camera itself was not placed inside of the MRI bore, there is not a need for the more costly MRI compatible systems. The camera is attached to an MRI safe IV pole via a Loc-Line modular hose to enable positioning changes. The other components are also inexpensive and can be self-built. We also added the information about the camera details as required by the reviewer.

-Because I have used a very similar system myself, I know about the problem using a camera live-image in the MR scanner. Usually, the camera provides a fast, real-time image when the image is directly transferred to the monitor screen. This comes with the cost, however, that the video cannot be implemented into a running program, such as matlab. That is, the camera image is fast, but just a "raw" image, which cannot be switched on or switched off, for example. Because the cues were auditory, and because the patient was asked to close the eyes, I think that the authors chose exactly such a setup here. However, this setup seriously limits the potential applicability of the protocol. For a well-controlled experiment, one would also need a visual condition WITHOUT leg movement, by showing a video. One would also need a leg movement condition with a similar fixation point compared to the visual condition, which would require showing a fixation cross instead of the video image. With this present setup, this cannot be provided. For such a setup, the camera image would have to be imported into e.g. matlab, but then, it would lose its timing accuracy. I therefore do not agree with the authors claims that this setup is ideal, or better than previous ones. I would ask the authors to please comment on all points mentioned above, and to include information about timing accuracy to back up their comments.

Answer: We thank the reviewer for pointing this out. This limitation is exactly why we decided to pursue our protocol as stated in the manuscript**.** The goal of this setup was capture the real time experience of moving subject's own leg. As pointed out, this is only possible by transmitting a high resolution of the leg movement in real time. If the goal of the protocol was not dependent on time accuracy, we would agree that more conditions (e.g. fixation and video) could provide more information. However, as discussed in the introduction, the possible mechanism underlying the effects of mirror therapy that we are evaluating is related to the fact that the mirror image helps to integrate the mismatch between proprioception and visual feedback.

- The authors do not provide any details on the quality of the camera image. A short video sequence has to be included into the manuscript submission, such that the reader can see the quality of the images used here. From my experience, MR-compatible camera images are usually of low quality, because they are very simple system without eg. brightness control. This is one reason why many researchers do not use camera images in the MR scanner. I myself for example have refrained from this option after trying different possibilities for many years. Please provide details.

Answer: We also added the information about the camera details as required by the reviewer. We confirm that the camera acquires 1080p image resolution images.

- The authors say in the discussion that their protocol is better than the at present available ones, particularly with respect to immersion. This is pure speculation. It is not clear whether the authors protocol evoked immersion. It is not clear how realistic the images looked. THe protocol was also not compared to previous protocols. I would ask the authors to remove this statement, and to provide a more balanced discussion of their results. It is a serious drawback of the study presented here that participants did not fill out a rubber-hand-like questionnaire where some aspects about the illusory character were assessed. Also this should be acknowledged.

Answer: We have modified the text accordingly to avoid the assumption that our protocol is better with respect to immersion. However, we still believe that seeing the participant's own leg image generates a more immersive feel than seeing a virtual animated representation of a leg. This is due in part to the fact that all participants have morphological differences. Therefore, it is plausible to consider that seeing your own body image will generate a more immersive feel than viewing a generic animation. Still, as mentioned by the reviewer, this is based more on speculation. The text has now been modified to reflect this issue.

Minor Concerns:

- In the introduction, the authors only reference work that provides evidence for the "distortion theory" of phantom pain, according to which distortions in the representation of the missing limb lead to phantom pain. However, there is work by the group of Prof. Makin showing the reverse: preserved topography relates to phantom pain. THis literature should be included.

> **Answer:** The most studied theory and the one in which the hypothesis of this study is based on is the theory of cortical reorganization. Therefore, this is the one most discussed in the introduction. However, we agree that it is important to mention all theories involved in the PLP process. We emphasize that this is a procedure paper and thus mechanistic discussions are beyond the purpose and goal of the this journal publication..

- the authors repeatedly say that they monitor the "leg". In fact, participants only move the foot. Please replace "leg" by "foot" throughout the manuscript to avoid confusion

> **Answer:** Although participants are only moving the foot, this was a point we discussed in the beginning of our protocol and we believe that using the word “leg” was more appropriate as we wanted the patient to have the impression that they were moving the whole leg. Therefore, the wording was important after discussing with patients and members of the research staff. We have added a phrase explaining this rational in the revised manuscript.

- the here introduced protocol is different from the usual mirror therapy setup in the sense that here, the patient does not see his/her own leg in the correct perspective. He/she sees the leg from below, not from above. In my view, this is a serious drawback of this protocol. Ideally, the mirror would be mounted in a way that the correct perspective is preserved, as for example done in the design of Keysers et al. 2004 Neuron. I would ask the authors to discuss this issue, and to perhaps try such an arrangement in the MR scanner

> **Answer:** We agree with this potential limitation. Participants usually perform leg mirror therapy in a sitting position, placing the mirror between the legs and looking at the mirror image from the side. This orientation is not possible in the fMRI scanner. In this protocol, patients must lay down to be scanned and the camera is placed on the side of the non-amputated leg and thus observing in real time the leg from that side (in the same way if the participant was sitting). This is in contrast to the study of Keysers et al. 2004 where participants were touched on their legs or observed movies/video images of other people’s limbs or objects being touched.

- Stimulus-associated movements are a serious problem in this design. Could the authors please provide the movement parameters of the MR images in one further figure? Knowing in how far this protocol evokes movements of the head is one important factor for considering / not considering using this protocol

Answer: As previously discussed, controlling for head movements is an important limitation and concern as with any fMRI protocol that uses a task requiring movement. For this protocol, we decided not to use head constrains during the images acquisition. However we used foam pads to stabilize the head and avoid movements. From an analysis standpoint, we used dynamic stabilization to minimize motion artifacts, and motion correction was performed as discussed in the analysis section of this protocol:

"Volumes with motion above 0.9mm in any direction should be marked with FSL's motion outlier detection processing stream and mathematically "scrubbed" from the final analysis. If more than 25 percent of the volumes are designated for removal, the whole acquisition should be excluded from the total dataset".

- Could the authors please add more info on the MR images acquired (sequence used, which filter, which segmentation etc.)

Answer: All subjects will undergo an MRI scanning protocol using a 3T Philips Achieva system and a thirty-two channel head coil (Center for Biomedical Imaging at Boston University School of Medicine in Boston, MA). The anatomical T1 images have a repetition time (TR) of 3 s and an echo time (TE) of 6.8 ms. Each functional acquisition run provides 182 volumes with a voxel size of 3 mm (isotropic resolution) recorded with TR=2 s, TE=28 ms.

Controlling for potential motion related artifacts is of considerable importance in this study as patient and/or novice individuals unfamiliar with the MRI environment can show greater magnitudes of head motion during image acquisition. This includes prospective motion correction (PMC) which will be used for functional scan acquisitions. PMC measures changes in geometry related to subject motion during the acquisition of a dynamic series and makes real-time adjustments based on the updated measurements. Second, the acquisitions will be processed for automated identification and removal of motion-related artifacts, volumes with motion above 0.9 mm in any direction should be marked with FSL's motion outlier detection processing stream and mathematically "scrubbed" from the final analysis. If more than 25 percent of the volumes are designated for removal, the whole acquisition should be excluded from the total dataset.

Reviewer #4:

Manuscript Summary:
This submission describes a protocol for assessing mirror therapy fMRI correlates with the subject experiencing real-time visualization via video projection inside the scanner. This is an improvement over previous techniques, which may have not matched well in real-time synchronization of movements or felt realistic to a participant. The protocol is aimed at people with leg amputations and phantom limb pain but there would be implications to use this in other contexts like post-stroke pain.

Major Concerns:
None.

Minor Concerns:

1 - Some justification for the particular choice of leg movements (ie. why just flexion and extension of the foot?) and timing of tasks (why 20 seconds per condition?) during the experiment would be helpful. Was there any dose-finding work? Similarly, the need for a mock scanner beforehand would suggest that there may have been some challenges with subjects beginning with the "real" scanner - any information on how the use of the mock scanner improved the protocol would be helpful.

Answer: For the type of the movements, we did several tests in order to get the best signal possible with the least amount of head motion possible, allowing good data quality collection. For fMRI, block design experiments are easy to carry out. The blocks were always of a fixed length, and lasted 20 sec depending on the timing of stimulus. In the case of physiological movements such as the one used here, 20 sec was enough to see clear modulation of the hemodynamic signal.

The mock scanner allows the participant to familiarize themselves with the environment and the protocol leading to improved and more accurate performance at the moment of the real experiment. Therefore, they can be better accommodated and comfortable for the real fMRI experiment.

2 - Authors state this is "real-life immersive sensation provided to the patient" - have subjects actually been asked about their impressions of the protocol and in comparison to others?

Answer: There is no interview or questionnaire to address real-life immersive experience. However, several patients reported VR immersion, including during protocol setup in which we used healthy controls. We have added further explanations as part of the protocol limitations in regard to this aspect.

3 - line 111 - change "emersion" to "immersion"

Answer: The correction was made.

4 - Since the person with the mutation here is not being treated clinically, would not use the term "patient" - maybe "participant" or "individual"

Answer: All the terms were substituted.

5 - Any differences to account for if amputation is above or below the knee?

Answer: The level of amputation should not interfere with the protocol execution since all participants use the non-amputated leg to perform the movements and have no visualization of the residual limb or phantom leg.

Submission ID #:

Editor Name:

Videographer name:

Film Date:

Authors and Affiliations:

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Title: Real Time Video Projection in an MRI for the Characterization of the Neural Correlates Associated with Mirror Therapy treatment for Phantom Limb Pain

Authors, please fill out the brief questionnaire below.

A. Will you require assistance with video microscopy, such as filming a complex dissection or microinjection technique (Y/N, please specify steps by number. Also, please list make and model of your microscope)? ____N______

B. Does your protocol include detailed, step-by-step, descriptions of software usage (Y/N, please specify steps by number)? ______N___

C. Which steps of your protocol will viewers benefit most from having filmed? Please list 4-6 steps_step: 3.2., step: 4.2., step: 4.9. and step: 5.3. _____

D. What is the single most difficult aspect of this procedure? Positioning the mirror inside the scanner as to allow the patient to observe the reflection adequately.

1. Introduction (Schematic Overview and Interview)

A. Schematic Overview (read by voice talent at JoVE):

Authors, please select from "Procedural Narrative" or "Conceptual Narrative" and complete the statements below. Please do not add additional steps. Then, attach your finished graphic overview. See accompanying instructions for details and examples.

Procedural Narrative:

The overall goal of this procedure is to more accurately characterize the neural correlates of mirror therapy in phantom limb pain patients, that is PLP patients. (Intro).

This is accomplished by the following steps. Step one: ensure that the participant does not have any known contraindications to MRI scanning and provide a prerecorded audio to make sure that they are able to understand and follow the instructions provided during the scanning procedure. **(P1).**

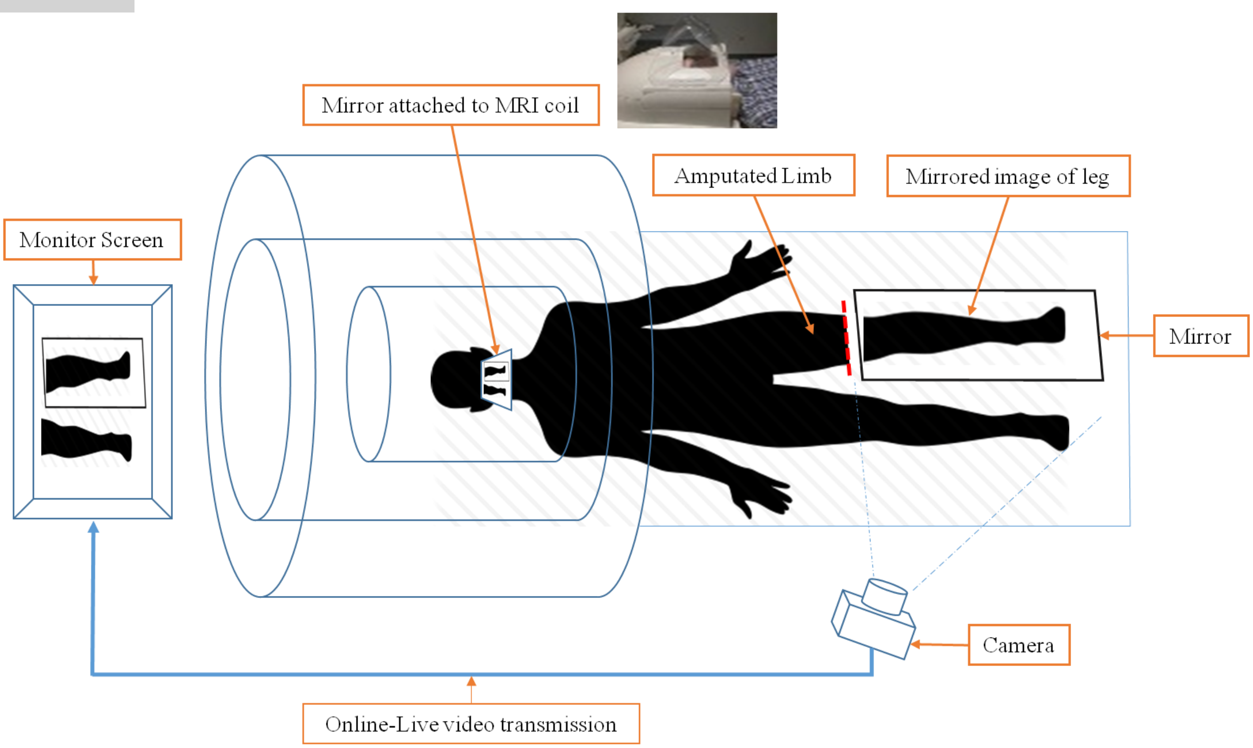
Step two: position the patient as comfortably as possible in the scanner bed where he or she should be lying supine with a single piece MRI compatible horizontal mirror between his or her legs. This mirror should be supported by triangular stand to avoid contact with any part of the patient's body. **(P2).**

Step three: place a MRI compatible digital camera on an adjustable tripod stands near the patient's intact leg to provide real-time video transmission. **(P3).**

Step four, the final step: Begin with an anatomical MRI and adjust the machine settings to each patient and then while the functional MRI scan is being performed play the recordings to the patient instructing him or her to complete the specific behavioral tasks. **(P4)**.

Ultimately the mirror attached to the MRI coil will allow patients to watch the mirrored leg movements in real time without moving their heads. **(P5).**

Paste a copy of your graphic overview here. The original file should be **adobe illustrator (preferred) or powerpoint** (see instructions) and should be uploaded through your online submission on the JoVE website.



B. Interview: (Said by you on camera. Don’t forget to smile!)

Authors: Below are statements we would like you to complete that are complementary to the information contained within the schematic overview. Only one statement should be chosen and completed per author who will be on camera demonstrating the protocol. In addition to choosing and filling out the appropriate statement, please enter the name of the individual who will say each line. **If individuals will be doing the demonstrations but not speaking in the introduction, please use statement 1.8 to introduce these demonstrators (ex PI introducing students).

Protocol (read by voice talent at JoVE):

Authors: In order to ensure that your protocol can be filmed in a single day, the protocol text must be limited to 30 steps – each step being defined as 3 lines of 12 pt text in our formatting style below. This amounts to 3 pages of protocol text. The scope of the scripted protocol text should include only those aspects of the procedure that require visualization in order to be well understood.

2. Materials (film the materials)

2.1. For this protocol you will need the following items: an MRI scanner, two MRI compatible mirrors; a large one placed between the patient's legs as well as a small one to place on the head coil. Additionally, you will need sandbags, an MRI compatible digital camera, a tripod for the camera, a computer controlled system and a monitor to place in the back of the scanner bore.

3. Subject placement

3.1. Before proceeding to the MRI it is critical to make sure the patient has no known contraindications to MRI scanning for example metal implants, aneurysm clips or severe claustrophobia.

Initially you will explain to the patients exactly what they should expect during the experimental procedure. The patients will then listen to a recording with instructions to follow during the scan.

3.2. Patients may first practice during a mock scan to become familiar with the task as well as the scanner environment. The mock scanner is similar in every way to the real MRI scanner but without the active magnet.

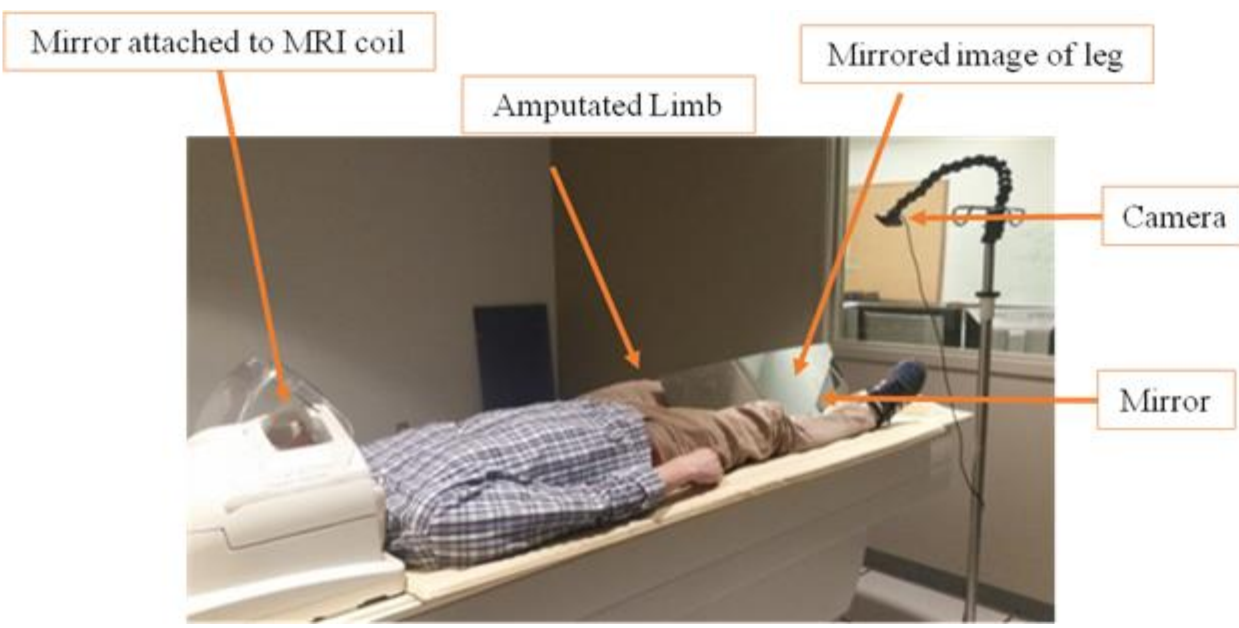
3.3. Before entering the scanner room patients must remove their prostheses as well as any metal objects they might be wearing on their heads or bodies for example watches or jewellry. The MRI technician will make sure that patients have no metal that might put them at risk.

3.4. All patients are transported to the scanner room using a MRI safe wheelchair to avoid falling out.

3.5. After that the patient's will transfer themselves to the MRI scanner bed.

3.6. After the patient is lying comfortably in the supine matter on the scanner bed a single piece MRI compatible horizontal mirror is placed between his or her legs and adjustable arm is then positioned to point the camera at the mirrored leg.

3.6.1. FIGURE 2



3.7. The large mirror is positioned between the legs at an angle about 45° depending on patients height and amputation level. The goal is to cover the stump to make it invisible to the videocamera. Sandbags are used to keep the mirror at the correct angle.

3.8. A smaller mirror is positioned on the head coil angled at 45° at eye level. This mirror allows the patient to visualize the mirrored leg image directly without moving their head while lying completely inside the scanner bore.

3.9. A MRI compatible digital camera is mounted on a tripod stand near the intact leg. This camera will transmit real-time video images of the mirrored leg movements to a computer control system that then projects the video to a monitor near the patient's head, so she or he can view the mirrored leg movement.

4. Preparing the experiment

4.1. The patient will undergo a four-minute anatomical scan first followed by four runs of functional acquisitions while he or she performs the tasks. Each run lasts six minutes.

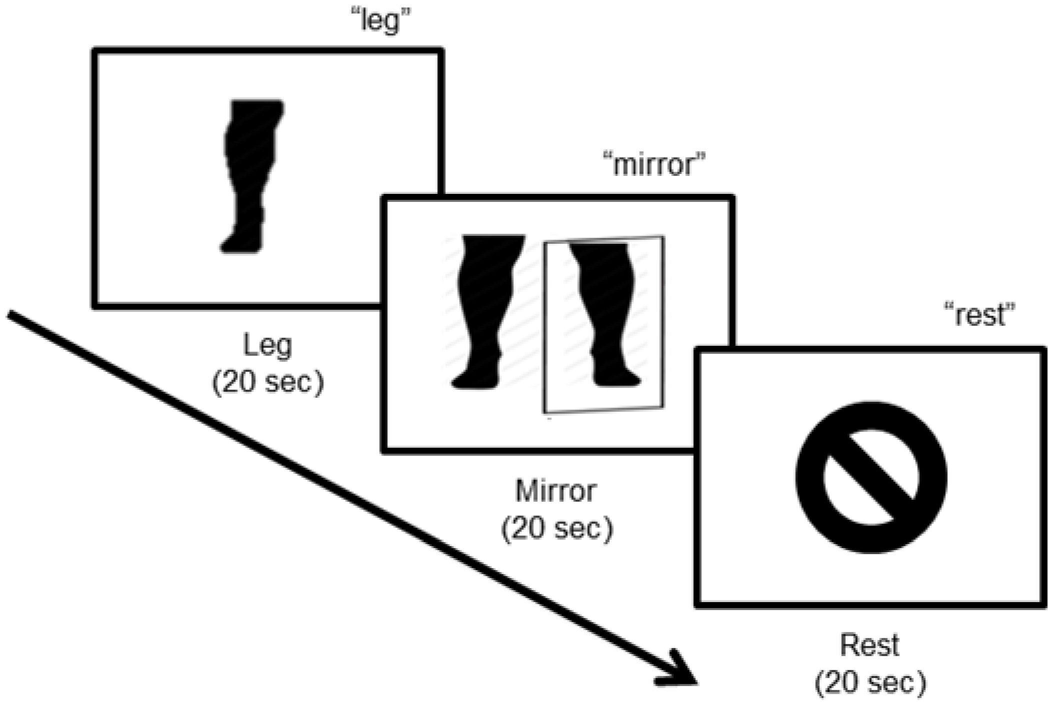
4.2. During the scans the patient wears sound isolating MRI compliant headphones which emit a series of auditory cues instructing the patient to perform the given behavioral task. The following commands are used 1) leg, 2) mirror and 3) rest. Additionally the investigator says start and end at the beginning and end of the experimental run.

4.3. The patient has already been instructed on hearing the word “leg” to follow the tapping sound presented in the audio with the eyes closed he or she will tap the foot at a rate of one tap for two seconds for a total of 10 taps in 20 Seconds.

4.4. On hearing the second command mirror the patient has to continue tapping his foot at the same rate this time while looking at the display showing the mirrored image of the two legs. Again this would be at a rate of 10 taps in 20 Seconds.

4.5. On hearing the third command rest patient should stop moving his or her foot and lying motionless with both eyes closed.

4.5.1. FIGURE 3



5. Scanning and Data collection

5.1. Data is collected in a single session for each patient and the entire scanning procedure last approximately 30 minutes.

5.2. The investigators take notes of any unwanted movements. Between the runs they can ask the patients to keep the right pace and do the correct movements.

5.3. After the procedure is finalized the data is transferred to an encrypted flash drive and stored in secure location in the facility.

6. Analysis

6.1. A longitudinal analysis design is used comparing baseline and posttreatment data. The FSL software package and processing stream will be applied.

6.2. Volumes with motion above 0.9 mm in any direction are identified with FSLs motion outlier detection processing stream and mathematically scrubbed from the final analysis. If more than 25% of the volumes are designated for removal the whole acquisition is excluded from the total data set.

6.3. A region of interest - ROI - analysis is used. The primary ROI is defined structurally using FreeSurfer's Desikan Atlas of the primary sensory motor cortex and refined with the subject specific functional activation during the leg versus rest condition at the baseline scan.

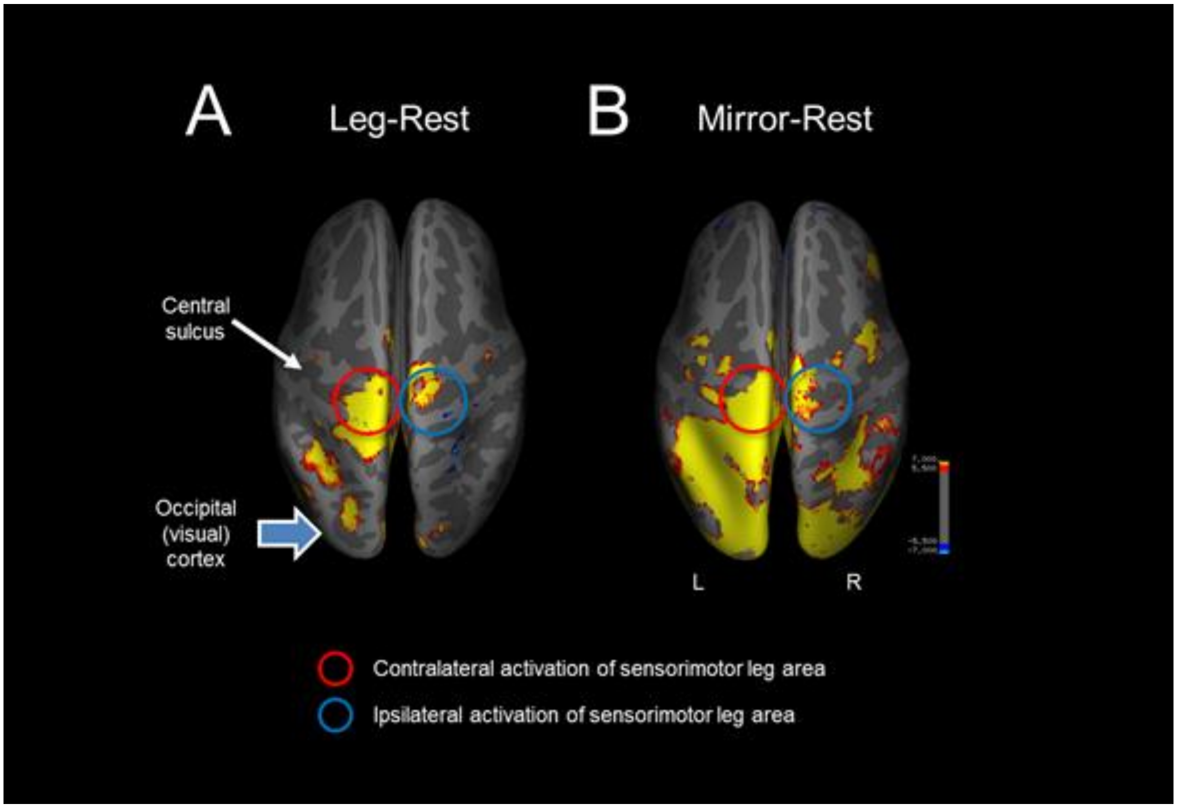
6.4. This ROI is then reflected on the homologous area of the other hemisphere. This is the ipsilateral primary sensorimotor representation of the intact lower limb. The secondary ROI is the entire – bilateral- occipital visual cortex as defined by the anatomical Desikan atlas.

7. Representative tDCS Results.

7.1. Patient reported that the experience immersive and the video images is lifelike. Therefore this real-time video projection process can generate sensations associated with conventional mirror therapy.

7.2. We expect that the leg condition that is the foot tapping task will lead to robust activation of the sensorimotor cortex representing the intact leg compared to the rest condition. However we also expect to see a less pronounced activation of the sensorimotor leg area representing the amputated leg.

7.2.1. Figure 4 A



7.3. The mirror condition also shows robust contralateral as well as some ipsilateral activation of the cortical leg sensorimotor area compared to the rest condition. Additionally, robust cortical activation is seen posteriorly in the visual cortical areas associated with viewing the mirrored leg.

7.4. The activation patterns described represent the baseline condition; that is prior to beginning therapy. These initial responses serve to define regions of interest - ROIs - and allow for comparison after the therapeutic protocol is completed in each individual.

INSTRUCTIONS FOR AUTHORS:

Please ensure that the representative results narration is appropriate and correctly describes your images, movies, or figures. Our editors have ensured that the results are written in our format.

We consider this section a critical aspect of the video, because here is where you provide validation for your experiments. For example, if this is a cell culture preparation, this section is where the video will show your cells at various time points following culturing. If this is an imaging prep, then this part is where you will show examples of your imaging experiments.

Please limit the extent of narration to no more than 2-3 lines of text per image or movie file being described. Figures with multiple panels submitted with the original protocol should be broken up so that each panel is a separate image. Like the schematic, each image or movie file supplied in the results should be referenced by annotation in parenthesis, however for the results, the specific filename should be given in parenthesis.

Below is an example of results text:

EXAMPLE REPRESENTATIVE RESULTS

5. Evaluation of Morpholino Injection and Knockdown

5.1 Representative results of both morpholino injection and mRNA injection are shown here. The uninjected control at 48 hours post fertilization looks normal, as expected
-LAB MEDIA: 0123_PIname_Figure1.tif (Replace 0123 with your jove video #)

5.2 However, embryos injected with the morpholino heg_e3i3_egfr1, which knocks down Heg isoforms
containing the first of two EGF-like repeats, exhibit brain edema.
-LAB MEDIA: 0123_PIname_Figure2.tif

5.3 Injection of heart of glass mRNA also produced an obvious phenotype. At 24 hours post fertilization,
the heads of the uninjected controls look normal
-LAB MEDIA: 0123_PIname_Figure3.tif

5.4 Conversely, some of the embryos injected with the mRNA exhibit cyclopia
-LAB MEDIA: 0123_PIname_Figure4.jpg

Please visit the following URL to see an example of how the results will look when complete:
http://www.jove.com/index/Details.stp?ID=1597

5. Conclusion (said by authors on camera)

Authors: Below are statements we would like you to complete that summarize and conclude the video. Only one statement should be chosen and completed per author who will be on camera demonstrating the protocol. In addition to choosing and filling out the appropriate statement, please enter the name of the individual who will say each line.

5.1. Camila Pinto: After watching this video, you should have an adequate and sufficient understanding of the necessary steps required to set up all the equipment to perform mirror therapy inside an MRI scanner.

5.2. Faddi Saleh: This protocol describes a novel, feasible procedure that allows investigators to more accurately characterize the neural correlates associated with mirror therapy in individuals with Phantom Limb Pain.

5.3. Camila Pinto: We could answer additional questions regarding brain organization after a limb amputation following the steps of this protocol using other neurophysiological measurements or imaging techniques.

5.4. Lotfi Merabeth: A challenge associated with this approach is the risk of generating excessive head motion artifacts, given that the leg must be moving repletely inside the scanner. Excessive head motion may compromise image data quality. In this regard, it is important to plan ahead and implement a variety of strategies to mitigate this possibility. These include training the participant in a mock scanner to carry out the task without excessively moving their head, making sure that the head is secure yet comfortably restrained and implementing motion detection and correction strategies during the acquisition and data analysis phase respectively.

5.5. Felipe Fregni: Given the method to implementing the experimental setup is relatively simple, this approach may allow the evaluation of the mirror therapy effects not only in limb amputees, but also in other conditions, such as stroke or spinal cord injury where mirror therapy is already commonly used in clinical practice.

Provided Media

Authors, Please list all images, movie files, or 3-D rendered animations that can be included in the video per editor's request. The step in the script/video where these images will be inserted should be specified. For example:

6.2 – *0123_PIname_Figure1.tif* - dual color imaging of tumor angiogenesis at 40X
6.2 – *0123_PIname_Figure2.tif* - dual color imaging of tumor angiogenesis at 100X

Formats: For static images we prefer .tiff files at dimensions of at least 720X480 pixels and 300 dpi. The higher resolution, the better. Likewise any exported movie files should have at minimum these dimensions and be rendered to .mov, .mp4, or .avi files.

Insert your media file names here.

General Preparation

It's critical for a smooth and organized shoot that all reagents are accounted for, in advance.

Any overnight or long incubation steps should be recognized and specimens/samples be prepared in advance so that prior steps can be recorded and shooting can continue with pre-prepared specimens/samples.

All tubes/flasks should be pre-labeled neatly before we arrive.

Ex. Luciferase assay done in 96 well plates should be labeled with negative/positive control wells and experimental samples are labeled accordingly.