Ann Ist Super Sanità 2019 | Vol. 55, No. 4: 408-412
DOI: 10.4415/ANN_19_04_18

Refinement techniques in non-human primate neuroscientific research

Luca Bonini

Dipartimento di Medicina e Chirurgia, Università degli Studi di Parma, Parma, Italy

Abstract
Non-human primates (NHP) are widely considered an essential model for biomedical research because of their close genetic, anatomo-functional and cognitive similarities to humans. These same reasons also raise particular ethical concerns for the unavoidable harm caused to these animals, in particular to those involved in neuroscientific studies. Besides reducing the number of animals needed to the absolute minimum, it is therefore essential to implement procedures allowing, at the same time, to minimize the harm to the animals and maximize the quality and ecological validity of the data. Technological progresses have made possible, for example, to self-train monkeys in their home cage with positive reinforcement techniques and to adopt various types of telemetric systems for wirelessly recording neuronal activity in freely behaving animals. Example of full application of these techniques are still very limited in the literature, but different recent international projects and pioneering studies are paving the way for turning to the use of new technologies to get a more "ethically acceptable" NHP neuroscientific research.

Key words
- behavioural monitoring
- enrichment; housing
- Macaque monkeys
- neurophysiology
- wireless recording

INTRODUCTION

Non-human primates (NHP) are widely considered a still un-Replaceable model in different fields of biomedical research because of their close phylogenetic relationship with humans [1]. Although more reliable, predictive and economic – and hence overall better - methods without the use of animals are evermore available for many fields of research, NHPs remain crucial especially in basic neuroscientific studies [2]. Indeed, the understanding of complex behaviours requires, at the same time, non-human models of sufficient complexity to exhibit these behaviours and the possibility to investigate their physiological bases up to the single neuron level, thus implying invasive methods that cannot apply directly to humans. The lack of alternatives in this specific field of research makes it critical to implement fully the remaining 2Rs: Reduction and Refinement.

Concerning Reduction, the most widely accepted standard in NHP neuroscientific studies requires to provide substantial evidence of a given mechanism in one subject and then to replicate the findings in a second animal. Recently, the execution of anatomical tracing studies in the same animals previously used for physiological experiments [3] is contributing further to reduce the number of animals needed with no relevant impact on cumulative severity. At the same time, this approach increases the amount and usefulness of information gathered from each single animal, allowing to integrate the contribution of multimodal data. In fact, a largely established trend indicates that, in Italy, less than 1% of the NHPs used each year in biomedical research (corresponding to less than 10 animals) are employed for basic research [4].

Being Reduction fully implemented at the highest level in this field, investing on the Refinement of the procedures in a lifetime perspective assumes paramount importance. Refinement measures do not only allow minimizing the harm to the animals but can also maximize the quality and validity of the collected data [5]. Three main domains of application of refinement measures can be identified: 1) husbandry and housing, 2) techniques and procedures, 3) data collection technologies. In the following sections, I will review the available evidence in each of these domains, in the attempt to trace a possible roadmap for future directions toward a more ecologically-relevant and ethically acceptable use of NHP in basic neuroscience research.

REFINING HUSBANDRY, HOUSING AND ENVIRONMENTAL ENRICHMENT

The Italian law in force (DL.vo 26/2014), which adopted the EU Directive 63/2010 in a more restrictive form, prohibits the breeding of all non-human primate species destined to biomedical research, forcing Italian scientists to import animals from abroad. This leaves outside of full national control the breeding and husbandry history of imported animals and adds the unnecessary, stressful event of long-range transportation in NHPs' lifetime experience.

Address for correspondence: Luca Bonini, Dipartimento di Medicina e Chirurgia, Università degli Studi di Parma, Via Volturno 39/E, 43125 Parma, Italy. E-mail: luca.bonini@unipr.it.

Considerable progresses have been made in defining optimal housing conditions and devising a large variety of environmental enrichments, which significantly improve the well-being of captive NHPs. Primates are highly social animals, and must be housed at least in pair, allowing direct interaction with their peer and possibly visual and auditory contact with other subjects in the facility. The size of the enclosures must be adequate, and possibly larger than the minimum imposed by the law for a pair of subject of any given species. The space available should allow animals to walk, climb, jump, and in general to express the largest variety of their specie-specific behavioural repertoire. To better fulfil this requirement, environmental enrichment plays a pivotal role: foraging devices can be used to encourage monkeys to leverage their cognitive and motor skills to move in and explore the environment to look for food. Wooden structure, robs and swings can induce monkeys to climb and explore the environment from elevated positions, which positively contribute to the animals' feeling of security. In addition, providing coarse grain sawdust bedding or a bark substrate where spilling seeds or other small food items greatly contributes to maintain the variety of manipulative, foraging activities that primates typically entertain in their natural environment. Highly trained technical and research staff is a crucial element to ensure proper monitoring of NHPs' welfare. Attention should be paid as to whether and how individual animals appreciate specific enrichment items, when and for how long to use each one, and possible interaction between enrichment items provided to individual animals and the requirements of the type of study in which animals are enrolled. Previous works provide exhaustive reviews on these issues [6, 7].

Cognitive enrichment also plays a pivotal role in improving NHP welfare, and it is now widely recognized as an opportunity to facilitate specific research activities. Although it may sound strange, meeting NHPs' psychological and physical needs does not necessarily imply replicating the natural conditions in which they live in the wild. Indeed, NHP are extremely flexible and can adapt to a variety of conditions, even in the wild (e.g. when they adapt to live close or within human urban settlements). On these bases, it has been proposed to shift from the concept of "ethologically appropriate" to "functionally appropriate" environments. This perspective emphasizes that non-natural stimuli such as computer testing, music, videotapes, television viewing, positive human-animal interactions and even training can provide enriching opportunities and have positive outcomes on multiple measures of psychological well-being of NHPs [8], and at the end may also facilitate the acquisition of skills useful for the research project in which animals are involved.

REFINEMENT IN BEHAVIOURAL TECHNIQUES AND TRAINING PROCEDURES

One of the most widely established practice in NHP handling and training is the exclusive use of positive reinforcement techniques: these same techniques, originally conceived and developed in the field of behavioural psychology, are employed successfully in many other species like dogs, birds, marine mammals and even humans. By leveraging operant conditioning, it is possible to train both simple behaviours, like moving from a cage to another, and complex ones, like tool use [9] and cognitive tasks [10].

The same techniques, carefully applied by skilled and highly trained personnel, can be used to habituate NHP to enter and sit in a primate chair, which is often necessary for most of cognitive and behavioural neuroscience studies. Different methods have been developed to habituate NHP to approach and finally enter a primate chair or transport box without the traditional use of rigid collars and poles. Although these latter devices may not significantly impair the animals' welfare if appropriately employed by trained personnel [11], accumulating evidence indicate the possibility to automatize chair-training of NHPs in order to make them enter a primate chair and stick out their head for neckplate placement with positive reinforcement techniques controlled by computerized systems. This minimizes the need of human intervention and hence the possibility to introduce human error in the procedure, making possible to drastically reduce animals resistance to entering the chair [12].

Computer-aided unsupervised procedure have been also successfully employed to automatize self-training with positive reinforcement of monkeys directly in their home enclosure [13, 14], even without food or fluid restriction [15]. These methodologies have numerous advantages: they do not require a constant interaction between the animal and the caretaker or the experimenters, the progresses are self-paced by the animal ensuring an optimal individualized learning speed based on automatic updating of the task conditions and, altogether, these positive features enhance animals' motivation and welfare.

An important aspect which applies to every training procedure with NHP is that, in contrast to what a naïve view may suggest, daily training on a regular basis does not negatively impact on objective ethological measures of NHP well-being [16]. Instead, it may facilitate the progress of the training and contributing to reduce possible animal stress because it increases the animals' feeling of predictability and sense of control over the situation. This is even more true if regular routines are maintained in conjunction with the above reviewed novel methods, which may contribute to improve the welfare of the trained animals by reducing their stress, the time taken to achieve the desired level of performance and the need to limit their possibility to keep a sense of control over the situation.

REFINEMENT IN DATA COLLECTION TECHNOLOGIES

The experimental investigation of brain functioning at a system level can be carried out with various methodologies but, in both human and non-human primates, usually it requires studying still brains of awake and collaborative subjects. In the case of monkeys, temporary immobilisation of the head is often necessary to make it possible the neural recordings. This implies that, once habituated to entering a primate chair and being moved from the facility to the laboratory, monkey's head must be stabilized with different techniques that prevent it from moving during the neural recordings. Non-invasive head immobilization systems have been developed in addition to the traditional surgically implanted head posts [17]. Some of these new, non-invasive devices can also allow NHP to engage in the head-fixed task setting on a voluntary basis [18]. Nonetheless, one of the most important progresses that could dramatically contribute to increase both animal welfare and data quality is represented by the possibility to abandon head restriction and to study brain activity in moving brains of unconstrained animals, using telemetric techniques. Several methodological and technical papers have been published in the last 15 years showing the feasibility of wireless recording and emphasizing the considerable opportunities offered by this technique. However, wireless recording technologies have been very rarely employed in NHP, and in most of these few cases, they have been focused on the attempt to implement brain-machine interfaces rather than to the investigation of important basic research questions concerning brain functioning. The reasons for this apparent suspicion toward these techniques is that their development has not yet been paralleled by the introduction of appropriate systems for monitoring the animals' behaviour, rendering the experimental control an extremely challenging obstacle.

Nowadays, minimally invasive multielectrode systems are available to make it possible the recording of several individual units' activity simultaneously. These systems can be easily interfaced with various type of devices for wirelessly recording neuronal activity in freely behaving animals [19-21]. In addition, marker-less or hybrid (marker-based and marker-less) tracking systems are being exploited to increase the precision of behavioural monitoring in freely moving animals, in order to minimise the invasiveness of the procedures and allowing to collect data of higher ethological relevance. For example, Berger and Gail [22] have built a cage allowing to study wirelessly recorded neuronal activity during instructed reaches towards targets positioned in a 3D environment, where the animal could move without any body or head restriction while its movement was tracked with a real time video motion capture system. Besides improving animal welfare even during the experiments, these technologies can make possible to study the neural bases of complex behaviours completely inaccessible to scientific investigation as long as monkeys are constrained on a primate chair, such as communicative vocalization [20], walking [23] or wake-sleep related processes [24].

Ongoing projects carried out in our laboratories [25] have made possible to build an innovative setup and methodological approach to simultaneously study NHP behavior and brain activity during unconstrained, close-to-natural situations. To this purpose, we designed and built a transparent plexiglass enclosure in which the space can be easily manipulated and/or flexibly filled with different devices, such as food and non-food items, ropes and wooden structures, to elicit a wide variety of NHP ethologically relevant behaviors. We called this facility NeuroEthoRoom (NER) because it allows to investigate the neuronal correlates of ethologically relevant behaviours and cognitive processes in freely moving monkeys. A system of 8 IR-sensitive cameras set around the NER allows us to record NHP behavior with high temporal resolution (up to 100 Hz) from multiple viewpoints, allowing to automatically track monkey's position without the use of body-attached markers *(Figure 1)*. This new setup makes it possible to study a large variety of ethologically relevant behaviors in the NHP repertoire, which were impossible to investigate with conventional methods, and by integrating this technology with wireless neural recording systems synchronized with the video behavioral monitoring system the NER will allow us to correlate neural activity with NHPs' natural behavior.

Once fully developed, this approach will offer the possibility of investigating neurophysiological mechanisms underlying unconstrained social interactions between pairs of individuals tested simultaneously, or even among multiple members of a social group, paving the way to a truly social neurophysiology.

CONCLUSIONS AND FUTURE DIRECTIONS

Neuroscientific investigation traditionally pursued the goal of exploring the neural basis of cognitive functions and complex behaviours shared by human and non-human primates, using the latter as a model for a mechanistic understanding of how human brain works. To this purpose, it has been necessary to devise experimental settings enabling to get a simplified and more controllable version of the behaviours and processes observed and described in the wild or in unconstrained captive animals by ethologists, to the purpose of making possible the simultaneous recording of brain activity with the various available techniques. This has created two orders of problems: 1) constrained laboratory conditions raised ethical issues on animal welfare and 2) the results collected in these conditions could be only indirectly linked with behaviours or processes studied in evermore naturalistic settings.

The more and more refined techniques in data collection technologies described in this paper may reach a stage in which neural activity could be sampled from chronic neural implants and transmitted wirelessly at long distances with completely subcutaneous, minimally invasive devices, similar to those used for deep brain stimulation in parkinsonian patients. These systems may be used for a few weeks in non-purpose bred, captive NHPs, temporarily exploited for specific neuroscientific studies (impossible to be performed in humans) directly in open-field colonies. This approach, with the improvement of MRI-based anatomical techniques that do not require post-mortem analysis of brain tissue, may allow to explant and release the animals in their group with no consequence on their welfare at the end of the study.

Although this scenario may appear excessively unlikely for the near future, the developmental trend just started in the refinement of neural and behavioral data acquisition technologies will play a critical role in the next years in increasing the ecological validity of neuroscientific data as well as the range of behaviour and

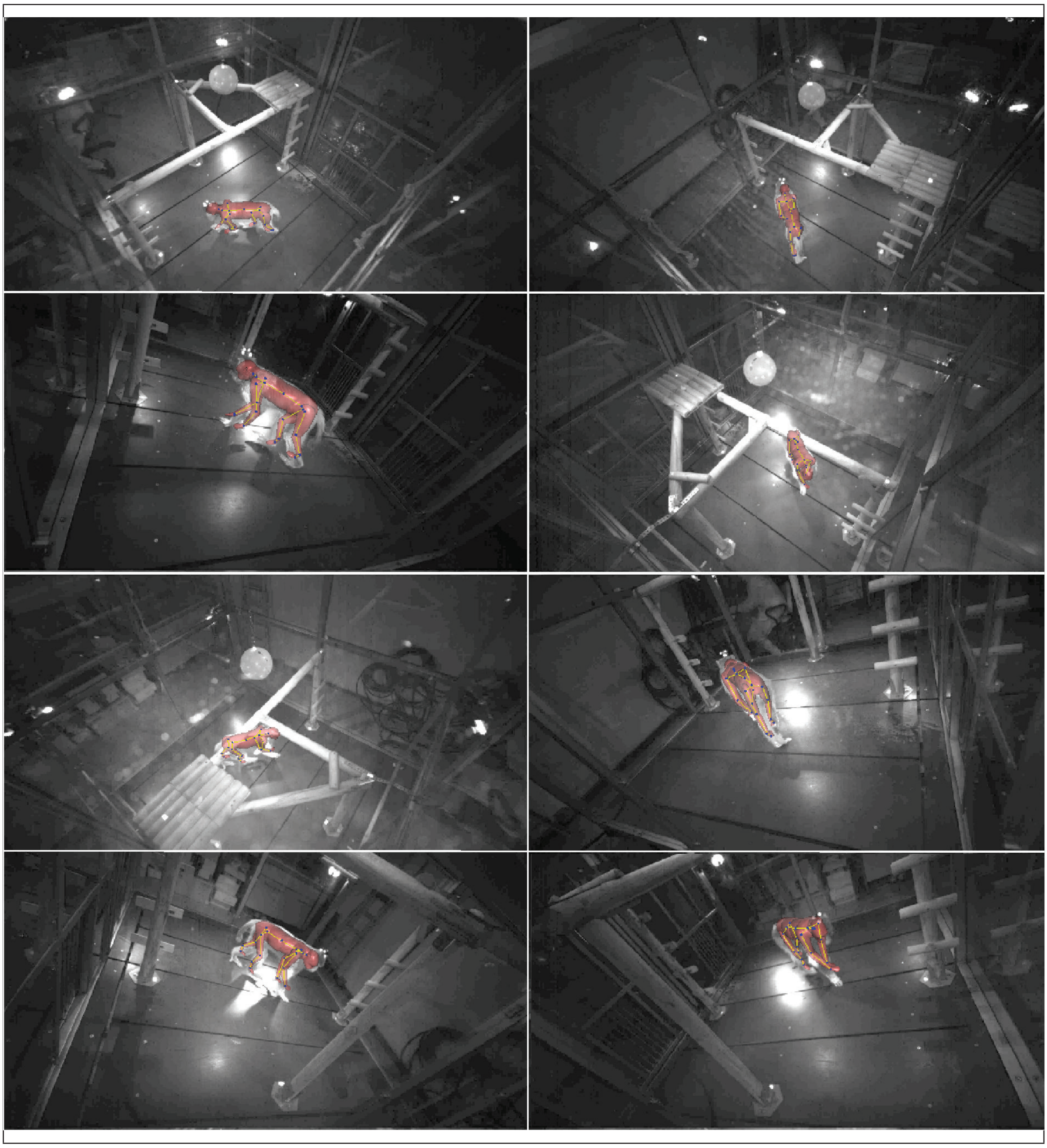

Figure 1
Example frame taken from 8 infrared video cameras showing a macaque monkey freely-moving in the NER overlapped with its avatar model. Note that the environment is large enough for allowing the monkey to move in 3D by walking, climbing, jumping, and potentially while interacting with its cage partner.

cognitive processes susceptible to neuroscientific investigation. This paradigmatic shift would be undoubtedly easier if paralleled by a transition from a multiplicity of small laboratories to the convergence of facilities, equipment and personnel into big primate centres, where a multidisciplinary neuroethological approach can make basic neuroscience research with NHP more ethically acceptable and hence more transparent and easy to communicate to the public opinion. All these achievements will be critical for ensuring a future to this "small but indispensable component of biomedical research" [2].

Conflict of interest statement

There are no potential conflicts of interest, financial or personal relationships with other people or organizations that could inappropriately bias this paper.

Submitted on invitation.
Accepted on 27 September.

REFERENCES

1. Scientific Committee on Health, Environmental and Emerging Risks. Final opinion on "The need for non-human primates in biomedical research, production and testing of products and devices (update 2017)". SCHEER; 2017.
2. Roelfsema PR, Treue S. Basic neuroscience research with nonhuman primates: a small but indispensable component of biomedical research. Neuron. 2014;82(6):1200-4. doi: 10.1016/j.neuron.2014.06.003
3. Lanzilotto M, Ferroni CG, Livi A, Gerbella M, Maranesi M, Borra E, Passarelli L, Gamberini M, Fogassi L, Bonini L, Orban GA. Anterior intraparietal area: A hub in the observed manipulative action network. Cereb Cortex. 2019;29(4):1816-33. doi: 101093/cercor/bhz011
4. Italia. Ministero della Salute. Comunicato 02 febbraio 2019. Dati statistici relativi all'utilizzo di animali a fini scientifici per l'anno 2017 (19A00632). Gazzetta Ufficiale – Serie Generale n. 28, del 2 febbraio 2019.
5. Wurbel H. More than 3Rs: the importance of scientific validity for harm-benefit analysis of animal research. Lab Anim (NY). 2017;46(4):164-6. doi: 10.1038/laban.1220
6. Vitale A, Manciocco A, Alleva E. The 3R principle and the use of non-human primates in the study of neurodegenerative diseases: the case of Parkinson's disease. Neurosci Biobehav Rev. 2009;33(1):33-47. doi: 10.1016/j.neubiorev.2008.08.006
7. Rennie AE, Buchanan-Smith HM. Refinement of the use of non-human primates in scientific research. Part II. Housing, husbandry and acquisition. Anim. Welf. 2006;15:215-38.
8. Bloomsmith MA, Hasenau J, Bohm RP. Position statement: "Functionally appropriate nonhuman primate environments" as an alternative to the term "Ethologically Appropriate Environments". J Am Assoc Lab Anim Sci. 2017;56(1):102-6.
9. Macellini S, Maranesi M, Bonini L, Simone L, Rozzi S, Ferrari PF, Fogassi L. Individual and social learning processes involved in the acquisition and generalization of tool use in macaques. Philos Trans R Soc Lond B Biol Sci. 2012;367(1585):24-36. doi: 101098/rstb20110125
10. Fischer B, Wegener D. Emphasizing the "positive" in positive reinforcement: using nonbinary rewarding for training monkeys on cognitive tasks. J Neurophysiol. 2018;120(1):115-28. doi: 101152/jn005722017
11. Scott L, Pearce P, Fairhall S, Muggleton N, Smith J. Training nonhuman primates to cooperate with scientific procedures in applied biomedical research. J Appl Anim Welf Sci. 2003;6(3):199-207. doi: 101207/S15327604JAWS0603_05
12. Ponce CR, Genecin MP, Perez-Melara G, Livingstone MS. Automated chair-training of rhesus macaques. J Neurosci Methods. 2016;263:75-80. doi: 101016/jjneumeth201601024
13. Calapai A, Berger M, Niessing M, Heisig K, Brockhausen R, Treue S, Gail A. A cage-based training, cognitive testing and enrichment system optimized for rhesus macaques in neuroscience research. Behav Res Methods. 2017;49(1):35-45. doi: 103758/s13428-016-0707-3
14. Berger M, Calapai A, Stephan V, Niessing M, Burchardt L, Gail A, Treue S. Standardized automated training of rhesus monkeys for neuroscience research in their housing environment. J Neurophysiol. 2018;119(3):796-807. doi: 101152/jn006142017
15. Tulip J, Zimmermann JB, Farningham D, Jackson A. An automated system for positive reinforcement training of group-housed macaque monkeys at breeding and research facilities. J Neurosci Methods. 2017;285:6-18. doi: 101016/jjneumeth201704015
16. Hage SR, Ott T, Eiselt AK, Jacob SN, Nieder A. Ethograms indicate stable well-being during prolonged training phases in rhesus monkeys used in neurophysiological research. Lab Anim. 2014;48(1):82-7. doi: 101177/0023677213514043
17. Adams DL, Economides JR, Jocson CM, Horton JC. A biocompatible titanium headpost for stabilizing behaving monkeys. J Neurophysiol. 2007;98 (2):993-1001. doi: 101152/jn001022007
18. Slater H, Milne AE, Wilson B, Muers RS, Balezeau F, Hunter D, Thiele A, Griffiths TD, Petkov CI. Individually customisable non-invasive head immobilisation system for non-human primates with an option for voluntary engagement. J Neurosci Methods. 2016;269:46-60. doi: 101016/jjneumeth201605009
19. Omer DB, Maimon SR, Las L, Ulanovsky N. Social place-cells in the bat hippocampus. Science. 2018;359(6372):218-24. doi: 101126/scienceaao3474
20. Roy S, Wang X. Wireless multi-channel single unit recording in freely moving and vocalizing primates. J Neurosci Methods. 2012;203(1):28-40. doi: 101016/jjneumeth201109004
21. Yin M, Borton DA, Komar J, Agha N, Lu Y, Li H, Laurens J, Lang Y, Li Q, Bull C, Larson L, Rosler D, Bezard E, Courtine G, Nurmikko AV. Wireless neurosensor for full-spectrum electrophysiology recordings during free behavior. Neuron. 2014;84(6):1170-82. doi: 101016/jneuron201411010
22. Gail A, Berger M. The reach cage environment for wireless neural recordings during structured goal-directed behavior of unrestrained monkeys. bioRxiv. 2018. doi: 101101/305334
23. Capogrosso M, Milekovic T, Borton D, Wagner F, Moraud EM, Mignardot JB, Buse N, Gandar J, Barraud Q, Xing D, Rey E, Duis S, Jianzhong Y, Ko WK, Li Q, Detemple P, Denison T, Micera S, Bezard E, Bloch J, Courtine G. A brain-spine interface alleviating gait deficits after spinal cord injury in primates. Nature. 2016;539(7628):284-8. doi: 101038/nature20118
24. Jackson A, Mavoori J, Fetz EE. Correlations between the same motor cortex cells and arm muscles during a trained task, free behavior, and natural sleep in the macaque monkey. J Neurophysiol. 2007;97(1):360-74. doi: 101152/jn007102006
25. Lanzarini F, Lanzilotto M, Bonini L. The Neuroethoroom: a new tool for neurobehavioral studies in freely behaving monkeys. SfN Abst 6122, Chicago (USA), October 19-23, 2019.