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Utility of Organotypic Slices in Parkinson's Disease Research

Fabio Cavaliere and Carlos Matute
*Neurotek-UPV/EHU, Department of Neuroscience and CIBERNED,
Bizkaia Technology Park, Zamudio
Spain*

1. Introduction

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disease of unknown etiology. The pathology involves a degeneration of dopaminergic neurons of the substantia nigra with consequent dopamine (DA) deficiency and neurodegeneration of striatal neurons.

The most effective treatments target compensation for the DA deficit. Among the different strategies applied, the most used is pharmacological substitutive treatment with levodopa (L-DOPA). Nevertheless, all current treatments may be considered palliative and not curative.

Here we review the latest advances in research and preclinical investigation in cell restorative therapy by using slice tissue cultures as a model of PD. This chapter describes in detail the main organotypic models used to study the cellular and molecular mechanisms leading to dopaminergic degeneration. In particular, we move from the simplest organotypic co-cultured model of the substantia nigra-striatum-cortex to the unique slice of nigro-striatal slice used to study endogenous neurogenesis or viral vector-mediated overexpression of α -synuclein in midbrain slices. We discuss the possibility of using such models to induce adult neurogenesis by combining pharmacological treatments or for preclinical studies in cell transplantation.

The strategies in place thus far to restore the dopaminergic system in PD are pharmacological treatments, such as administration of L-DOPA, cell restorative therapy by gene therapy, or cell therapy by stem cell grafting. The main pharmacological treatment for PD symptoms today is the administration of L-DOPA, especially in the first stages of disease. L-DOPA, a DA precursor, acts to counteract the DA deficit in neurons. However, the beneficial effect of the drug lasts only a few years with disease progression and loss of efficacy, followed by onset of secondary effects such as on-off phenomena, dyskinesia, and psychosis. Moreover, recent studies in an animal model (Muller et al., 2004) suggest a toxic effect of prolonged L-DOPA treatment; thus, pharmacological investigation is devoted to studying new compounds or new therapeutic strategies. Inhibitors of an alternative metabolic route of DA such as entacapone and tolcapone are also used in pharmacological treatment (Linazasoro, 2008).

The administration of several neurotrophic factors has been intensively investigated in a rat model as a possible therapeutic strategy for PD (Grandoso et al., 2007). In particular, glial

cell line-derived neurotrophic factor (GDNF) has been shown to exert prominent action on dopaminergic neurons by enhancing their survival and stimulating the outgrowth of DA fibers (Kirik et al., 2004). As noted, current pharmacological therapy for PD is considered only palliative and not curative, reflecting the necessity for new strategies and new therapeutic targets.

The principal targets of gene therapy in PD are replacement of tyrosine hydroxylase (TH) striatal neurons and blocking of neurodegeneration of the nigrostriatal pathway. The latter can be accomplished with local expression of neuroprotective neurotrophins such as GDNF. One promising approach to gene therapy for PD is the combined use of craniotomy as a delivery system with viral vectors as a gene expression system. Injection of viral vectors for the expression of neurotrophic factors in primate models allows local production of therapeutic genes. Nevertheless, the single injection into the human or animal brain of either adeno-associated virus (AAV) or herpes simplex virus results in an inflammatory reaction leading to astrogliosis and demyelination (Dewey et al., 1999; McMenemy et al., 1998). Both AAV and retrovirus permanently and randomly integrate into the host genome, but neither AAV nor retrovirus crosses the blood-brain barrier (BBB). Therefore, it is necessary to administer the virus via craniotomy and by intracerebral injection, a very invasive approach. However, the most intense expression of the therapeutic gene is generally limited to the injection site, owing to limited diffusion of the virus within the brain. Current limitations of brain gene therapy approaches can be circumvented with the development of a transvascular delivery as a support for gene therapy. However, this approach would require the formulation of plasmid DNA in a way that allows the exogenous gene to cross the BBB and enter the brain via the transvascular route after an intravenous injection.

Cell therapy for PD is based on neural stem cell transplantation intended to give rise to new dopaminergic neurons. The *in vitro* features of stem cells include the ability to (1) proliferate in a 3D free-floating system called neurospheres, (2) exhibit self-renewal, (3) generate a large number of progeny through the transient amplification of a population of progenitor cells, and (4) retain their multi-lineage potential over time. These cells must generate new cells in response to injury or disease, create synapses, and integrate into a preexisting neuronal network. Cells that satisfy all of these criteria are defined as stem cells, whereas those cells that satisfy some but not all of the functional criteria listed above and are already committed to one specific lineage are defined as progenitor cells. From a clinical point of view, cell therapy in PD must satisfy the following objectives: (1) the grafted cells must survive and form connections; (2) the patient's brain must integrate and use the grafted neurons; and (3) the grafts must induce measurable clinical improvement (Lindval & Bjorklund, 2004; Meyer et al., 2010).

Cell regeneration can be obtained also by pharmacological activation of endogenous neurogenesis. Generation of newborn neurons in the adult mammalian brain occurs throughout life in the subventricular zone (SVZ) or in the subgranular zone of the hippocampus. In particular, the SVZ cover an interesting role in new cell regeneration due to the proximity with striatum. Neurogenesis can occur in brain regions which are damaged due to pathological conditions; in this case, the local environment influenced by cellular stress plays a crucial role in modulating the mechanisms of proliferation, migration and differentiation. Adult neurogenesis can be sustained, directly, by activating mechanisms of cell regeneration (e.g. with neurotrophic factors), or by the clearance of local negative environment (e.g. blocking excitotoxicity and apoptosis) (Vergni et al., 2009)

Despite problems, the use of cell therapy for the replacement of dead neurons results an attractive strategy for the treatment of PD. Here we review different *in vitro* experimental

models used to study the cellular mechanisms of PD and highlight the feasibility of such models for each of the use described above (pharmacological treatment, gene therapy or cell transplantation).

2. The advantage of using brain organotypic cultures

The necessity to cultivate slice tissues begins from the needs to find out a valid and reliable model between the monocultures and *in vivo* models. The organotypic culture resembles the *in vivo* model better than dissociated cultures. In fact, individual cells are in tight contact with each other, maintain their organotypic architecture, and preserve neuron-glia interactions, tissue-specific transport, and ion diffusion systems. Moreover, the organotypic cultures represent an efficient cellular model to overcome the *in vivo* impediments of the BBB. The strength of organotypic cultures in neuroscience research has been the opportunity to cultivate for weeks or months CNS tissue, giving open accessibility to complex cellular systems. Organotypic cultures are ideally prepared from P3-P10 animals (rats or mice) even if few attempts have been made to cultivate organotypic slices from adult CNS tissues. Young postnatal animals already possess essential cytoarchitecture, are easily handy -respect to the embryonic tissue- and nerve cells survive explantation more readily -respect to the adult slices. The advantage of use organotypic cultures derives from their versatility in experiments that require long-term survival, such as live recording (Zhang et al., 2007; Lacar et al., 2010) or pharmacology (chronic drug application) as well as electrophysiology.

Many different procedures have been proposed to maintain slice tissues from CNS in culture, but the most successful was the "roller tube" technique. This technique, finally characterized more in detail by Gaehwiler (1988), was developed on the basis of experiences based on multitude of works with explants culture.

In roller tube cultures (Fig. 1A), the tissue is embedded in a plasma clot and attached on a glass coverslip. The coverslip with the embedded slice tissue are located in a tube that undergoes continuous slow rotation in a cell culture incubator. The oxygenation is maintained by continuous exchange of liquid-gas interface generated by the slow rotation. The technique was successively modified several time (e.g. Braschler et al., 1989; Andres et al., 2005) but the roller tube technique always maintain a very thin cultures (from an initial 400 μm to about 50 μm) with consequential preferential use for experiments that require optimal optical conditions (e.g. electron microscopy or electrophysiology).

At the beginning of 90's Stoppini and colleague (1991) published a new method to cultivate organotypic slices. In this method brain slices were placed on a semiporous membrane and cultivated at the air-liquid interface (fig. 1B). The absence of clot facilitates the studies of synaptic reorganization being a useful tool to study plasticity and sprouting already during the first days of culture. The real advantage of this technique is that cultures are easily prepared and offer great advantages when a 3D structure is desired (from an initial 400 μm thickness, slices are cultivated up to 100-150 μm). The air-liquid interface has become a key instrument to study the adult neurogenesis. Organotypic cultures match the tri-dimensional space where neural progenitors migrate to reach maturation *in vivo*. In the paper by Vergni and colleagues (2009) the authors ideally represented the slice culture comprising subventricular zone (SVZ), as the spatial extension to elaborate a mathematical model to describe neuroblast activation and migration following oxygen and glucose deprivation.

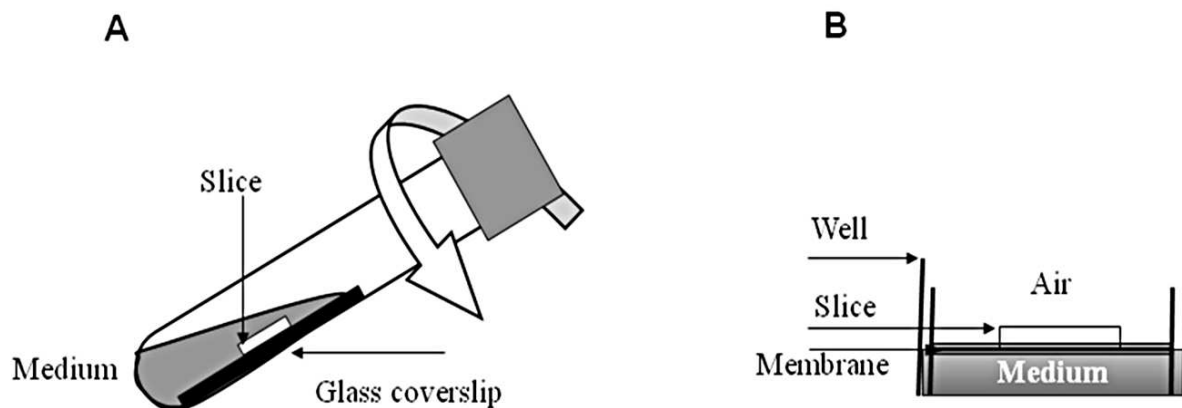


Fig. 1. **Roller tube (A) and semiporous membrane (B) methods used to cultivate organotypic cultures.** In both cases slices are cultivated in air-liquid interface, generated by tube rotation (A) or by the membrane (B) (see text for explanation).

3. Organotypic models for parkinson's disease

The first organotypic models used to study the nigrostriatal pathway were developed as co-cultures of slices from the substantia nigra with cortex and striatum (Ostergaard et al., 1996; Plenz & Kitai, 1998). These organotypic co-cultures, cultivated with using the roller tube method as well as a semiporous membrane, after several days in culture developed dopaminergic fibers connecting the substantia nigra with the striatum. Preservation of function of the newborn fibers was demonstrated by electrophysiology whereas the anatomical connection was shown by immunofluorescence. These organotypic models were used to study the role and the modulation of glutamate in dopaminergic development (Gramsbergen et al., 2002; Ostergaard et al., 1996; Plenz & Kitai, 1998). By confocal laser scanning microscopy, Plenz and Kitai demonstrated that the glutamate system modulated the dopaminergic innervations of the striatum. Activation of glutamate receptors generated sprouting of dopaminergic fibers and increase of innervations in the striatum. First was hypothesized that activation of glutamatergic receptors could induce neurogenesis but no generation of new neurons was observed in the substantia nigra. Increase of striatal neurons innervations was explained as a trophic effect generated by the selective activation of the metabotropic receptors of the mGluR group I, as specific mGluR I antagonists inhibited the dopaminergic innervation in the striatum. Results are sustained also by the mRNA expression of mGluR5 and mGluR5 receptor (Testa et al., 2005; Romano et al., 2005). Because both drugs were added to the medium, no conclusions could be drawn as to the site of drug action. In this case the stereotaxic application of the drug (see below Stahl et al., 2009) may account for a more precise evaluation of the mechanism.

The relevance of the co-culture was addressed later with studies of the effect of donor age on dopaminergic innervations (Gramsbergen et al., 2002). Because the model is composed of three different slices (co-culture), the age of the tissue donor is relevant to slice preservation and dopaminergic innervation. Of interest, it was found that the addition of brain-derived neurotrophic factor (BDNF) had a trophic effect and stimulated an increase in TH immunoreactivity in the striatum as well as protection from apoptotic death. BDNF also induced an increase in cell generation, possibly of progenitor cells, expressing TH immunoreactivity. These observations indicated that BDNF may sustain cell viability and dopaminergic differentiation after grafting of immature DA neurons.

PD in these slices can be modeled by treating organotypic cultures with toxins, selective for DA nigro-striatal degeneration (6-hydroxy DA-6-OHDA, rotenone, MPTP). As in animal model, 6-OHDA in organotypic cultures drives to neuronal death mediated by oxidative stress (Saner et al., 2007) inducing selective degeneration of dopaminergic neurons. However, this toxin doesn't produce extra-nigral pathology or Lewy body-like inclusions (Lane et al., 2007). In order to set up an organotypic culture as a model of PD it is important to take in account how to apply the toxin. Evaluation of neuronal death is different depending on the method of 6-OHDA application (Stahl et al., 2009). If toxin is applied directly in the medium it generates uniform DAergic cell death without accounting for natural variation occurring among slices, whereas local application of the drug at the tissue surface of main dopaminergic nuclei (substantia nigra pars compacta, ventral tegmental area and retrorubral field) of organotypic cultures, cause a precisely localized cell death resembling *in vivo* stereotactic model (Stahl et al., 2009).

Recently, we proposed an innovative organotypic model to study nigrostriatal degeneration and adult cell regeneration (Cavaliere et al., 2010). The presence of the nigrostriatal network with the cortex and subventricular zone (SVZ) in a single slice made possible the study of the mechanisms of nigrostriatal degeneration as well as the mechanisms of adult neurogenesis from the SVZ. This type of slice was made by cutting in a single plane the region between the ventral mesencephalon and the cortex (Fig. 2).

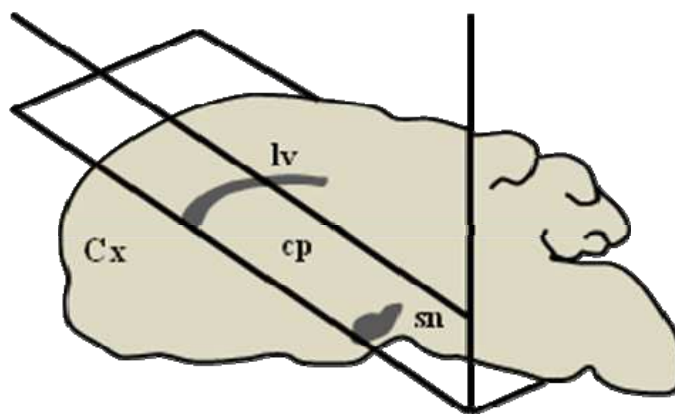


Fig. 2. **Schematic view of organotypic slice preparation preserving the nigrostriatal pathway.** Slices are cut (350 μ m) at 45° in a single plane to include the substantia nigra (sn), striatum-caudate putamen (cp), subventricular zone of the lateral ventricle (lv), and cerebral cortex (cx).

In this model, nigrostriatal degeneration can be obtained by mechanical transection of nigrostriatal fibers or chemically, for example by treatment with the DA analog 6-OHDA. In both cases, in agreement with the results presented by Gramsbergen and colleagues (2002), the degeneration of the nigrostriatal network induced classic PD features (e.g. α -synuclein inclusions, Halliday and McCann, 2008) and increase in TH expression in the neurogenic niche of the SVZ. It is still not clear whether the increment of TH in SVZ cells means an increment in protein synthesis or generation of newborn TH⁺ cells, but the functional meaning seems to be a response to the damage generated in the striatal area.

PD can also be modeled in organotypic cultures from cortex-striatum and SVZ (Cavaliere et al., 2005; Vergni et al., 2009; Tønnesen et al., 2011; Fig.3) in which dopaminergic inputs from the substantia nigra to the striatum are severed by slicing. This model is extremely useful

especially in studying neurogenesis induced after severe nigro-striatal degeneration. Tønnesen and colleagues (2011) used this model to study functional graft of multipotent stem cells in damaged striatum. It is well known that effective synaptic integration into pre-existing neuronal circuitry after stem cell transplantation results difficult to analyze despite integration in the host tissue and robust behavioral rescue (Parish et al., 2008). In this paper the authors overexpressed the gene *Wnt5a* into TH-GFP derived neurospheres to generate DA neurons. These cells were then transplanted in the cortex-striatum-SVZ organotypic model and patch clamp recording and optogenetic tools were used to study the functional properties and synaptic integration of transplanted DA neurons into host cells. Host brain tissue was modified genetically to respond to optogenetic stimuli. Briefly, optogenetic is the response, in milliseconds time-scale, of cells overexpressing photo-sensitive receptors, like channelrhodopsins (ChR) or halorhodopsin (NpHR). Cells modified in this way can be depolarized (in case of ChR) or hyperpolarized (in case of NpHR) when stimulated with blue or orange light respectively (Miesenböck G. 2009). Optogenetic stimulation of organotypic slices and patch clamp recording of GFP grafted cells revealed synaptic integration and bidirectional connection of grafted cells with cortex and striatum.

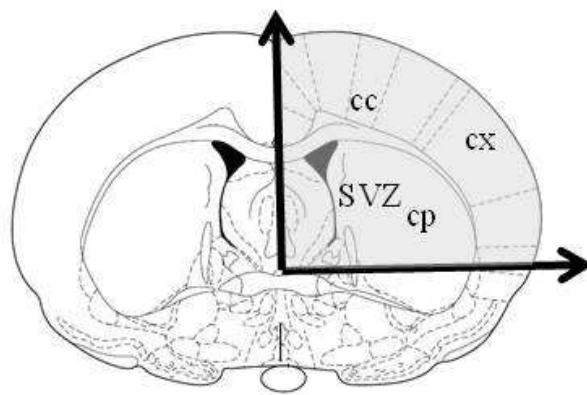


Fig. 3. **Schematic view of cortex-striatum and SVZ organotypic slice.** Slices are cut (350 μ m) coronally in a single plane to include the striatum-caudate putamen (cp), subventricular zone of the lateral ventricle (SVZ), cerebral cortex (cx) and corpus callosum (cc) and cultivated in air-liquid interface.

Organotypic cultures were used also to study the effect of α -synuclein on midbrain neurons. This model resembles the animal model obtained by viral transduction of human α -synuclein in rodents or non human primates (Kirik et al., 2002, 2003; Eslamboli et al., 2007). Differently from the methods that use drug delivery, this model doesn't address free radical damage and associated mitochondrial dysfunction, moreover the neurotoxin models are essentially non-progressive and do not replicate all aspects of the disease. This model takes advantage of the special property of recombinant vectors generated from adeno-associated viruses (rAAV) to transduce the nigral dopamine neurons with very high efficiency. In the organotypic model, midbrain slices were transfected with truncated α -synuclein (A53T) fused to EGFP (Zach et al., 2007). Virus exposure resulted in dramatic changes in neurite morphology with α -synuclein locally accumulated in distorted and swollen neuritis. Neuritic swelling was observed especially in long neurites originating from putative projection neurons transduced with truncated more than with the full-length α -synuclein, as already demonstrated in animal model and PD patients.

4. Advances of preclinical cell therapy

Different cell types have been used for experimental and clinical cell therapy, giving in all cases partial PD symptom improvement. Neurospheres from human fetuses have already been used for PD cell therapy (see Brundin et al., 2000, and Lindval & Kokaia, 2006, 2009, for a review). They consist of both multipotent stem cells and more restricted progenitors at different stages of differentiation. Kim and colleagues in 2006 performed a comparative study between neurospheres obtained from different fetal brain tissues, in order to check whether the neural stem and precursor cells have specific regional or temporal characteristics with regard to growth, differentiation, and region-specific gene expression. They found that isolated neurospheres from different CNS compartments expressed distinctive molecular markers of regional identity even if this regional pattern could be reversed by environmental factors.

Transplantation studies in animal models of PD have highlighted the main problems that arise from the stem cell grafts (Table 1). In line with this the preclinical use of organotypic tissue model to study mechanisms and cell regeneration in PD becomes worthy.

In most cases, grafted cells displayed many of the morphological characteristics of DA neurons, with expression of dopaminergic markers and reinnervation of the lesioned striatum. However, cell therapy presents secondary effects. First, the risk of teratoma from embryonic and fetal stem cell transplantation is relatively high. It has to be considered that direct implantation of mouse embryonic stem cells into the rat striatum gives rise to tumor generation in 20% of the cases (Freed et al., 2001). Second, in 15% of the patients transplanted with stem cells of fetal origin, post-operative dyskinesias have been observed in the "off" phase (Bjorklund et al., 2002). In fact, the most important problem with the use of human fetal stem cells, besides the poor availability, is the lack of standardization, which results in a high variability in the degree of symptomatic relief.

CELL	REINNERVATION	SYMPTOMS IMPROVEMENT	LIMITS	REFERENCES
Mouse ESC	Partial	Partial	Risk of teratoma Ethical considerations	Kim et al., 2002
Monkey ESC	Partial	Partial	Risk of teratoma Ethical considerations	Sanchez-Pernaute et al., 2008
Human ESC	Not detected	Partial	Risk of teratoma Ethical considerations	Cho et al., 2008
Rat BMSC	Fibers	Partial	Lack of homogeneous differentiation, no evidence of dopaminergic specialization, low integration into pre-existing neuronal network	Dezawa et al., 2004
EMT	Extensive	Partial	Ethical considerations, Lack of homogeneous differentiation	Winkler et al., 2000

Table 1. Stem cell graft in animal models of PD. ESC, embryonic stem cells; BMSC, bone marrow stem cells; EMT, embryonic mesencephalic tissue.

To overcome the secondary effects of using embryonic and fetal stem cells, several groups have used adult stem cells for transplantation located primarily in the SVZ of the forebrain and in the sub-granular layer of the dentate gyrus of the hippocampal formation (Vergni et al., 2009). However, newly generated cells with a neuronal phenotype can be generated also from adult tissues that are different from the brain. Because of the ease of their isolation and their large potential for differentiation, mesenchymal stem cells are among the first stem cell types to be introduced into clinical studies. Results on differentiation of mesenchymal stem cells derived from such tissues are still controversial; e.g., debate persists about how to isolate a homogeneous population of the cells with specific criteria from the bone marrow, how to expand them *ex vivo* without affecting their differentiation potential, and how to develop easy methods for quality control of the cellular-based products (Chen et al., 2006; Kassem, 2006).

In addition to the use of the correct cell type (Meyer et al., 2010) and the generation of a standard protocol, other scientific improvements are necessary for the development of a clinically practicable cell therapy in PD. One is the proper selection of patients. It is of primary importance to determine whether a cell graft can be affected by disease progression. Cell regeneration should be done especially in those patients who positively respond to L-DOPA and in those patients for whom the main pathology is a loss of DA neurons, more than debilitating symptoms like dementia or degeneration of the non-dopaminergic system. In this regard, PET analysis before the engraftment is critical to understanding the preoperative degeneration pattern.

5. Conclusion

Cell therapy used to generate new dopaminergic neurons possesses a high potential. Up to now, all of the therapies used to counteract the symptoms of PD are palliative and limited in their therapeutic impact. Furthermore, the lack of standardized protocols and absence of a correct selection protocol for patients in the clinical trials result in a high variability in the degree of symptomatic relief. More efforts to surmount this variability and to achieve success in restorative therapy have to be made in this direction. The selection of a correct model to study the cellular and molecular mechanisms and for preclinical investigations represents a promising strategy to achieve a successful protocol for animal and human cell therapy.

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Parkinson's disease (PD) is characterised clinically by various non-motor and progressive motor symptoms, pathologically by loss of dopamine producing cells and intraneuronal cytoplasmic inclusions composed primarily of α -synuclein. By the time a patient first presents with symptoms of Parkinson's disease at the clinic, a significant proportion of the cells in the substantia nigra have already been destroyed. This degeneration progresses despite the current therapies until the cell loss is so great that the quality of normal life is compromised. The dopamine precursor levodopa is the most valuable drug currently available for the treatment of PD. However for most PD patients, the optimal clinical benefit from levodopa decreases around five to six years of treatment. The aim of the chapters of this book is to work towards an understanding in the mechanisms of degeneration and to develop disease modifying therapies.

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InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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