*Brief Report*

# Direct Chromosome Preparation Method in Avian Embryos for Cytogenetic Studies: Quick, Easy and Cheap

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**Abstract:** Avian cell culture is widely applied for cytogenetic studies, the improvement of which increasingly allows for the production of high-quality chromosomes, essential to perform both classical and molecular cytogenetic studies. Among these approaches, there are two main types: fibroblast and bone marrow culture. Despite its high cost and complexity, fibroblast culture is considered the superior approach due to the quality of the metaphases produced. Short-term bone marrow cultivation provides more condensed chromosomes but nonetheless is quicker and easier. In the search for a quicker, cheaper way to prepare metaphases without losing quality, the present work developed a novel, widely applicable protocol for avian chromosome preparation. Twenty-one bird embryos from distinct families were sampled: Icteridae, Columbidae, Furnariidae, Estrildidae, Thraupidae, Troglodytidae and Ardeidae. The protocol was based on a combination of modified fibroblast culture and bone marrow cultivation, taking the advantages of both. The results show that all species consistently presented good mitotic indexes and high-quality chromosomes. Overall, the application of this protocol for bird cytogenetics can optimize the time, considering that most fibroblast cultures take at least 3 days and often much longer. However, our protocol can be performed in 3 h with a much-reduced cost of reagents and equipment.

**Keywords:** Aves; cell cultivation; chromosomes; metaphases; method

**Citation:** Barcellos, S.A.; de Souza, M.S.; Tura, V.; Pereira, L.R.; Kretschmer, R.; Gunski, R.J.; Garnero, A.D.V. Direct Chromosome Preparation Method in Avian Embryos for Cytogenetic Studies: Quick, Easy and Cheap. *DNA* **2022**, *2*, 22–29. https://doi.org/10.3390/dna2010002

Academic Editor: Shin-Ichiro Hiraga

Received: 5 November 2021
Accepted: 17 January 2022
Published: 26 January 2022

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## 1. Introduction

Since the early 1900s, bird chromosomes have been investigated using classical and molecular approaches. Despite the fact that avian cytogenetics play an important role in evolutionary studies, less than 10% of all bird species have a karyotype description so far, and nearly all of these are only partial karyotypes [1]. Birds have a bimodal karyotype and a ZZ/ZW sex chromosome system [2]. One of the most remarkable characteristics in their karyotypes is the large number of microchromosomes, which encode a high rate of important genes [3]. Regarding diploid numbers, they display a wide range, from 40 to 142. Despite this variation, more than 50% of birds have between 78 and 82 chromosomes [1].

Working with wild birds is always challenging due to the limitations associated with animal sampling [4]. Even though some samples can be collected from birds in captivity, it is still hard to do this in some species without negatively affecting their health [5].

Avian cytogenetic studies require cell cultures that provide high-quality metaphases for chromosome analysis, as well as the application of classical and molecular techniques, such as karyotyping, chromosome banding and fluorescent in situ hybridization (FISH). In

recent years, FISH analysis has played an important role in evolutionary studies, revealing chromosomal homologies and rearrangements [6].

Due to the large amount of microchromosomes and the difficulty in distinguishing them, cells need to be free from residual content such as cytoplasm, which could cause analysis issues. In general, fibroblast culture is the most commonly applied protocol to obtain high-quality avian metaphases [7–12]. However, some authors use short-term bone marrow cultivation to perform cytogenetic experiments [13–16].

In 1968, Sasaki, Ikeuchi and Makino [17] developed a fibroblast cell culture protocol in order to obtain bird metaphases. Although this protocol has been widely used, there are limiting factors for its application, such as expensive reagents and equipment, contamination risk and long-term culture, which can last several weeks. In addition, cell culture protocols with a few modifications such as temperature and time of cultivation have also been used [7–10]. Short-term bone marrow culture is a low-cost technique in comparison; however, the metaphase quality is typically poorer due to the accumulation of residual content such as cytoplasm and typically short, condensed chromosomes [18]. Another short-term cell culture that can be applied for this approach is the colchicinized embryo technique developed by Bloom and Buss [19] and Bloom with modifications [20].

The purpose of this study was to develop a low-cost, short-term cell culture for avian cytogenetic studies that combined the ease of bone marrow culture with the quality of fibroblast culture using fertilized eggs as a starting material.

## 2. Materials and Methods

### *2.1. Bird Embryo Cell Culture Preparation*

For this technique, 3 fertile bird eggs were used from individuals from distinct families of birds, i.e., Icteridae (*Molothrus bonariensis*), Columbidae (*Columbina picui*), Furnariidae (*Synallaxis frontalis*), Estrildidae (*Estrilda astrild*), Thraupidae (*Sporophila caerulescens*), Troglodytidae (*Troglodytes musculus*), and Ardeidae (*Butorides striata*), ranging from 10 to 15 days old. All samples were collected following the protocols approved by the Ethics Committee on the Use of Animals (CEUA 019/2020) and Biodiversity Authorization and Information System (SISBIO 61047-3/33860-2).

To begin with, the eggs were examined by candling, holding them in front of a bright light. Shells were cleaned by being wiped with 70% ethanol and were allowed it to air dry. Then, the egg air sac was found and marked with a pencil. Subsequently, the shell above the air sac was removed with sterile forceps, without touching the egg membrane. Then, the membrane was removed, exposing the embryo, which was placed into a sterile Petri dish with egg content. After that, the embryo was removed to another sterile Petri dish. To prepare the cell suspension, the embryo was washed with 2 mL of Hanks' balanced salt solution 1x, and then, we dissected the embryo head using a sterile scissor. The Hanks' solution was discarded, and the embryo was macerated (shown in Figure 1a). Then, a 5 mL syringe with a needle and pipette was used in order to help mix the embryo with 2 mL of trypsin 0.25% EDTA (shown in Figure 1b,c). Using a sterile pipette, the sample was placed into a 15 mL conical tube with 2 mL of trypsin 0.25% EDTA and mixed until complete tissue disaggregation was achieved, for approximately 10 min. Afterwards, 1 mL of fetal bovine serum was used to inactivate the trypsin solution. The content was placed into a new tube containing 10 mL of RPMI 1040 medium pre warmed at 37 °C and mixed. Then, 3 drops of colchicine 0.01% was added. The sample was incubated at 37 °C for 1 h, and then subsequently centrifuged for 10 min at 1000 rpm (room temperature). The supernatant was removed, and the cell pellet was suspended in 10 mL of hypotonic solution (0.075 M KCl) at 37 °C for 20 min. Finally, 1 mL of methanol and acetic acid (3:1) solution was added in order to fix the samples. The process was repeated 3 times with 10 mL of methanol and acetic acid (3:1), each at 1000 rpm for 10 min before storage at −20 °C (shown in Figure 1d). From start to finish, the whole protocol took less than 3 h.

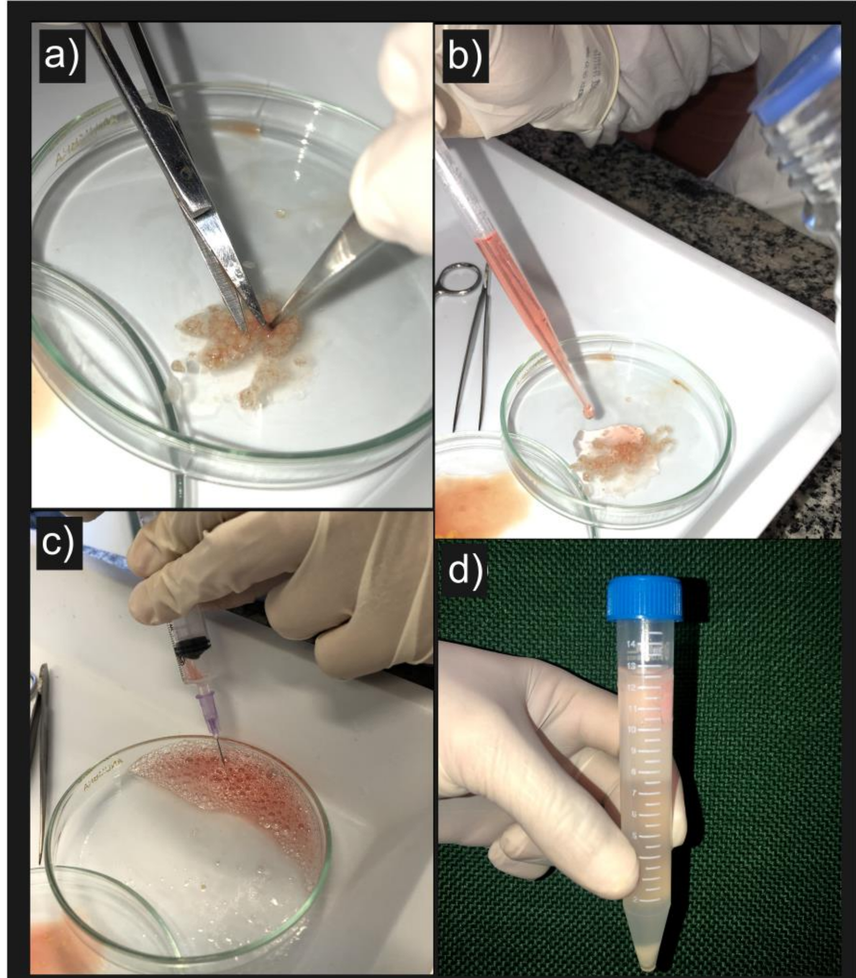


**Figure 1.** Some of the steps in preparing embryos for short-term culture. The embryo was macerated using sterile scissors and tweezers in a sterile Petri dish (**a**); 2 mL of trypsin 0.25% EDTA was transferred into the Petri dish using a sterile pipette (**b**); the embryo and the solution were mixed by using a 5 mL syringe with needle (**c**); after incubation and washes, the sample was ready to be stored (**d**).

Metaphases were stained with Giemsa 5% in phosphate buffer pH 6.8 for 5 min. Figures were photographed with an optical microscope (OLYMPUS DP53, Olympus Corporation, Ishikawa, Japan) and cellSens Imaging software (Olympus Corporation).

### 2.2. Fluorescent In Situ Hybridization (FISH)

In order to test the effectiveness of FISH analysis in these chromosomal preparations, we chose two sets of probes. Bacterial artificial chromosomes (BACs) from *G. gallus* microchromosomes (GGA12) and the Z sex chromosome (GGAZ) were respectively applied in the *B. striata* and *T. musculus* species, respectively. FISH experiments were performed in accordance with O'Connor et al. [21]. The results were photographed using an epifluorescence microscope (Olympus BX61, Olympus Corporation).

## 3. Results

Overall, all bird species sampled using this new embryo short-term protocol presented high-quality metaphases. Figure 2 displays three individuals sampled using this protocol. The chromosomal number found in these species was the same as previously cited in the literature, *S. caerulecens* 2n = 78, *E. astrild* 2n = 78 and *B. striata* 2n = 60 [22–26].

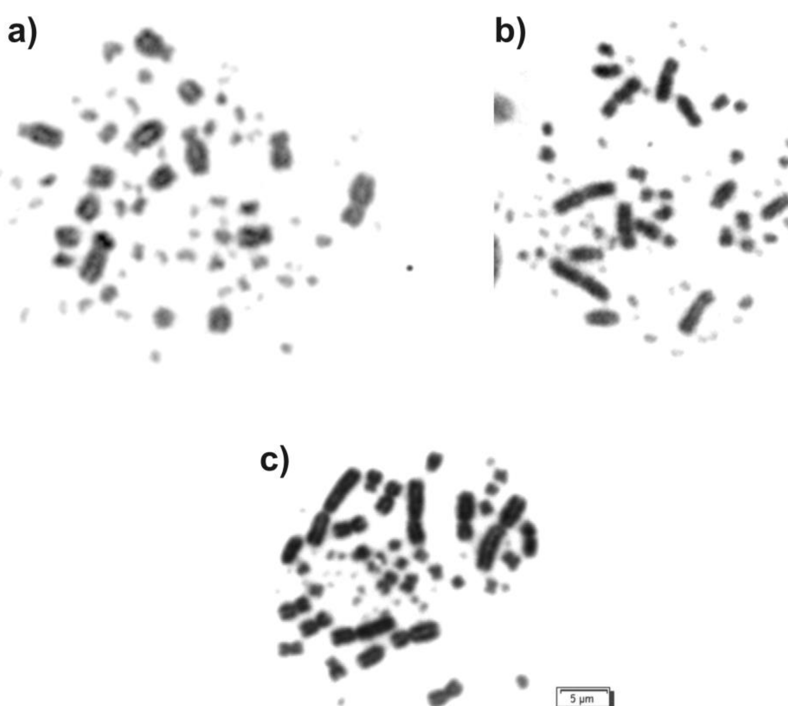


**Figure 2.** Conventional staining with Giemsa 5%. *Sporophila caerulescens* (**a**); *Estrilda astrild* (**b**); *Butorides striata* (**c**). Bar = 5 µm.

Although we did not perform specific statistical analyses of mitotic indexes, they were nonetheless high in all samples and more than good enough for our purposes. Several metaphases were present in each field of view for all samples.

The estimated time for all processes from the embryo disassociation to slide preparation was less than 3 h, with extra time being necessary for staining, FISH and microscopy.

The BAC probes were successfully hybridized, GGA12 in one microchromosome pair of *B. striata*, as well as GGAZ in the Z chromosome of *T. musculus*. FISH results are shown in Figure 3.

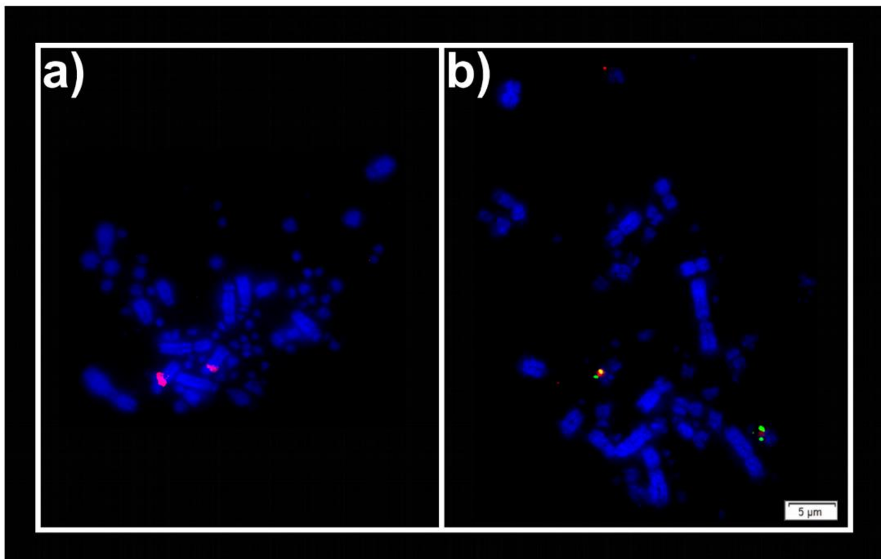


**Figure 3.** Fluorescent in situ hybridization analysis using BAC probes. (**a**) GGAZ (TGMCBA-270I9 Texas red) in *Troglodytes musculus.* (**b**) GGA12 (CH261-60P3 Texas red and CH261-4M5 Fluorescein isothiocyanate) in *B. striata*. Bar = 5 µm.

## 4. Discussion

The generation of suitable-quality metaphases in a reasonable time frame is the basis for all cytogenetic experiments. In this study, we combined the benefits of bone marrow cultivation techniques with fibroblast culture.

Long-term fibroblast culture from embryonic and non-embryonic cells such as muscle, liver, skin and lungs have been the most commonly applied technique for obtaining bird chromosomes [7,8,27–29]. However, these protocols require extensive time cultivation, large quantities of reagents and specific equipment, for instance, a $CO_2$ incubator [9,10].

The colchicinized embryo technique is also a good option for obtaining bird metaphases; however, as with the bone marrow cell culture, it contains residual cytoplasm, which can cause hybridization issues for FISH analysis [19,20,30,31].

This new method presents several benefits when compared to other types of cell cultures used for avian cytogenetics: (1) it is easier, cheaper and quicker than fibroblast cell culture protocols. Most of the previous fibroblast culture methods take more than 3 days, some of them more than 2 weeks, to obtain good quality metaphases [7–9,27,28]. By contrast, the method described here takes around 3 h in total; (2) one bird embryo provides numerous metaphases of high quality; (3) the method only requires a few items of equipment and reagents; (4) it has a low contamination rate. For fibroblast cultures, one of the major challenges is to avoid the contamination that can cause the loss of the samples. In contrast, our protocol can cope with this issue due to the short amount of cultivation time. A comparison table (Table 1) shows the advantages and disadvantages of the most common cell culture techniques in more detail.

**Table 1.** Comparison table between cell culture methods for bird cytogenetics.

| Techniques | Advantages | Disadvantages |
|---|---|---|
| Colchicinized embryo technique developed by Bloom and Buss [19] | • Good-quality chromosomes<br>• Short-term cultivation (approximately 2 h)<br>• Only requires a few reagents | • Accumulation of residual content in the metaphases |
| Fibroblast cell culture developed by Sasaki, Ikeuchi and Makino [17] | • High-quality chromosomes<br>• Metaphases free of cytoplasm | • Long-term cultivation (weeks)<br>• Requires many items of equipment and reagents<br>• Risk of contamination |
| Colchicinized embryo technique developed by Bloom [20] | • Good-quality chromosomes<br>• Short-term cultivation (approximately 1 h)<br>• Only requires a few reagents | • Accumulation of residual content in the metaphases<br>• Limited number of metaphases per embryo due to the absence of passages |
| Bone marrow developed by Garnero and Gunski [18] | • Short-term cultivation (approximately 1 h)<br>• Only requires a few reagents | • Accumulation of residual content in the metaphases<br>• Condensed chromosomes<br>• Limited number of metaphases per individual due to the absence of passages |

**Table 1.** *Cont.*

| Techniques | Advantages | Disadvantages |
| --- | --- | --- |
| Fibroblast (skin and liver) developed by Itoh and Arnold [9] | • High-quality chromosomes<br>• Possibility of increasing the number of metaphases using the passages | • Long-term cultivation (one week)<br>• Requires many items of equipment and reagents<br>• Risk of contamination |
| Cell culture developed by Tsuda and Umehara [10] | • High-quality chromosomes<br>• Possibility of increasing the number of metaphases using the passages | • Long-term cultivation (more than 48 h)<br>• Requires many items of equipment and reagents<br>• Risk of contamination |
| Method described in this paper | • Short-term cultivation (1 h)<br>• Only requires a few reagents and items of equipment<br>• High-quality chromosomes<br>• Contamination issues do not affect the quality of the culture | • Limited number of metaphases per embryo due to the absence of passages |

Bone marrow or fibroblast cultures that use the organs of adult individuals need to sacrifice the bird, which can be problematic if these animals were in the reproductive season [8–16]. Considering that in more than 80% of all bird species, both parents provide care, if one of the parents were to be sacrificed, its progeny would probably die [29,32]. Furthermore, there is no guarantee that the offspring would survive until adulthood due to nest predation and all of the difficulties in the maturation process [33–35]. Despite the high energy expenditure during reproduction, most females are still able to re-lay eggs in the same season in case the first attempt did not succeed [23,35–37]. Thus, this is another advantage of the bird embryo short-term culture protocol, as it tries to mitigate the environmental impact. Furthermore, the metaphases present a better quality than those obtained via short-term bone marrow culture [18].

Avian cytogenetics is concerned with genome reconstruction and comparative genomics [6]. Molecular cytogenetics has to keep pace with the number of species that are being sequenced, and rapid methods for chromosome preparation such as the one proposed are increasingly essential [38,39]. High-quality preparations with longer chromosomes allow for predicted chromosome fragments (PCFs) to be mapped more accurately, and cleaner preparations mean that cross-species hybridizations are more likely to work.

To this end, the present study provided a new method for cell culture that can be widely applied for cytogenetic studies in bird species.

**Author Contributions:** Conceptualization, S.A.B.; methodology, S.A.B., M.S.d.S. and R.K.; software, S.A.B.; validation, S.A.B., R.J.G. and A.D.V.G.; formal analysis, S.A.B. and R.J.G.; investigation, S.A.B. and M.S.d.S.; resources, S.A.B., R.J.G. and A.D.V.G.; data curation S.A.B.; writing—original draft preparation, S.A.B.; writing—review and editing, S.A.B., M.S.d.S., V.T., L.R.P., R.K., R.J.G. and A.D.V.G.; visualization, S.A.B. and M.S.d.S.; supervision, R.J.G. and A.D.V.G.; project administration, R.J.G. and A.D.V.G.; funding acquisition, S.A.B., R.J.G. and A.D.V.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (CAPES)—Finance Code 001.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA/SISBIO 61047-1) and approved by the Ethics Committee on the use of animals of Universidade Federal do Pampa (CEUA 019/2020, 30 November 2020).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in the article.

**Acknowledgments:** The authors would like to thank all members of the "Grupo de Pesquisa Diversidade Genética Animal" from UNIPAMPA and Darren K. Griffin, who kindly provided the BAC probes.

**Conflicts of Interest:** The authors declare no conflict of interest.

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